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Investigation of the Contribution of Type 1 Pili in Enterotoxigenic Escherichia Coli (ETEC) Pathogen-Host Interactions

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by
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ABSTRACT OF THE DISSERTATION

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Enterotoxigenic *Escherichia coli* (ETEC) are one of the leading causes of death due to diarrhea in children living in developing countries. ETEC are also the leading cause of diarrhea in travelers to developing countries lacking sanitation and safe drinking water. Unfortunately, there is no broadly protective vaccine available against these important pathogens. In order to cause infection, ETEC colonize the intestinal epithelium and secrete toxins, including heat-labile toxin (LT) and/or heat-stable toxin (ST). Efficient delivery of these toxins to the cognate receptors on target intestinal cells requires direct ETEC-host interactions. Earlier studies demonstrated that ETEC facilitate interactions with host by pathotype specific adhesive pili, termed as colonization factor (CF). However, recent studies demonstrate that in order to establish interactions with host,

ETEC engage multiple factors, including number of ETEC-specific virulence factors as well as factors those are highly conserved across different *E. coli*.

Detection of increased expression of type 1 pili encoding genes from cell adherent ETEC led to the hypotheses that these pili facilitate ETEC adhesion to intestinal epithelial cells and promote delivery of toxins to target cells. Using polarized culture of intestinal cell lines as well as human ileal enteroids, we observe that FimH adhesin of type 1 pili facilitate ETEC adhesion, antibody mediated or chemical inhibition of FimH interaction decrease ETEC adhesion and increased expression of highly mannosylated glycoprotein receptor of FimH enhance ETEC adhesion to host. Additionally, we find that FimH interactions augment delivery of toxins and are required for virulence, as we detect reduced fluid accumulation by *fimH* mutant in the rabbit ileal loop assays, a standard model of ETEC pathogenesis.

Although earlier studies reported that LT enhances ETEC-host interactions, the underlying enhancement mechanism is undefined. Using RNAseq and mass spectrometric analysis we demonstrate that LT enhances glycoprotein synthesis and increases surface presentation high mannose glycans. Additionally, LT increases expression of CEACAM6 receptor of FimH adhesin on intestinal epithelial cells. Interestingly, deletion of FimH-CEACAM6 interactions decreased ETEC adhesion to host, identifying a novel ETEC-host interaction. However, in contrast to the contribution of FimH in adhesion, deletion of FimH activity increased colonization of mice by ETEC. Interestingly, we detected increased expression of several adhesion related factors,

including PqiB, a homologue of Mam7 adhesin, by *fimH* mutant recovered from mice, signifying the importance of functionally complementary adhesins of ETEC. Together,

these findings propose a mechanistic explanation of LT-mediated enhancement of ETEC adhesion, and identify a novel ETEC-host interaction.

Collectively, data presented in the thesis demonstrate the contribution of type 1 pili in ETEC pathogenesis, notice previously unidentified FimH-CEACAM6 interactions of ETEC and identify a highly conserved putative Mam7 adhesin that may facilitate intestinal colonization by ETEC. Overall, these findings expand our understanding of ETEC pathogenesis and could supplement rational approach to broadly protective ETEC vaccine design.

CHAPTER ONE

Introduction: Type 1 pili in enterotoxigenic *Escherichia coli* infection

1.1 Global burden of enterotoxigenic *Escherichia coli* infections

More than 1 billion people are deprived of clean water (1) and 1 in every 3 people does not have access to even minimal sanitation (2). Consequently, acute diarrheal illness, mostly caused by ingestion of food and drink contaminated with pathogens transmitted via the *fecal*-oral route, is a leading cause of mortality and morbidity in children (3). In fact, it is the second leading cause of death in children under five years old (4), resulting more than 700000 deaths every year (5). Of the different causes, enterotoxigenic *Escherichia coli* (ETEC) are among major bacterial pathogens associated with severe diarrheal illness in children (6). ETEC are one of the major pathotypes of diarrheagenic *E. coli, and are* characterized by the production of heat labile (LT) and/or heat stable (ST) enterotoxins. Children less than 5 years of age, living in resource poor countries with compromised hygiene and sanitation, are particularly vulnerable to ETEC infections (7, 8). ETEC diarrhea is a leading cause of childhood morbidity and contributes substantially to delayed growth and development and malnutrition (9-11), which in turn increases the risk of repetitive diarrhea (12). ETEC also induce acute watery diarrhea in adults that can be severe and cholera-like requiring hospitalization (13).

Additionally, ETEC are frequently isolated pathogens in travelers with acute diarrhea. Travelers, including military personnel, from developed countries are at high risk of travelers' diarrhea when they visit countries with inadequate sanitation and safe drinking water. According to the U.S. Centers for Disease Control and Prevention around 10 million travelers get diarrhea each year (14, 15) and depending on the destination and

type of travel an attack rate of 29 cases per every 100 person-months was estimated by U.S. military (16).

Contaminated food represents another important vehicle of transmission for pathogens. Globalization of the food supply, which may inadvertently introduce pathogens to developed countries (17), poses a threat of foodborne diseases. In fact, every year, an estimated 1 in 6 Americans get sick from foodborne illness (18). Unfortunately, there have been a number of substantial ETEC outbreaks in the United States associated with contaminated food (19-23). Moreover, symptomatic and asymptomatic carriers including travelers, refugees and immigrants from developing countries may introduce these pathogens to developed countries (24), resulting in unavoidable emergence of ETEC pathogens in these regions (21-23, 25). Therefore, although endemic in the resource poor settings, ETEC infections present a global public health burden.

1.2 Enterotoxigenic *Escherichia coli* pathogenesis

Adhesion to the intestinal epithelial cells is critical for ETEC to become established at the site of infection (26) and for effective delivery of different effector molecules (27) that may modulate host responses. Several studies have demonstrated that the effective toxin delivery requires close association of bacteria to the host cell (27, 28). Interestingly, host cell contact was recently shown to modulate a variety of known and putative virulence genes (29, 30). Classically, ETEC deploy fimbrial virulence structures, known as colonization factors (CFs) for attachment to the intestinal epithelium (31). CF fimbriae are plasmid-encoded and both antigenically and structurally diverse. ETEC express more than 26 different CFs and an isolate may express one or multiple CFs on

the surface (31). Although CFs are important virulence factors, multiple studies reported that about half of the ETEC strains do not possess an identifiable CF (31, 32). Recent pathogenesis studies also suggest that these organisms require multiple virulence factors to effectively engage target intestinal epithelial cells and deliver toxins (33). These include at least two proteins with mucinase activity, including EatA and YghJ, which degrade intestinal mucin (34, 35), and EtpA, a novel secreted adhesin that binds to both the tip of the flagellum (36) and to the MUC-2 mucin (37), thereby acting as a molecular bridge between ETEC flagella and the host cell surface.

The biology of the toxins produced by ETEC has been defined in some detail. LT is an AB_5 enterotoxin consisting of pentameric B subunit (LT-B) and a single A subunit (LT-A) (38). Both monomeric LT-B and LT-A translocate to the bacterial periplasm via Sec machinery where LT-B molecules pentamerize and associate with LT-A to form the heterohexameric holotoxin. LT holotoxin is secreted across the outer membrane via a functional Type II secretion apparatus (39). The pentameric B subunit binds to GM1 ganglioside on the cell surface, triggering cellular uptake of the toxin. Following endocytosis, the catalytic LT-A subunit translocates through the vacuolar membrane and passes to the endoplasmic reticulum through the Golgi apparatus by retrograde transport and is activated by proteolytic cleavage (40). Once inside the cell, LT-A ADP-ribosylates the alpha subunit of G protein, a guanine nucleotide-binding protein that activates the catalytic unit of adenylyl cyclase (AC), resulting in permanent activation of AC (41, 42) and subsequent increases in intracellular cAMP (43). Increased cAMP in turn activates protein kinase A (PKA) resulting in phosphorylation of apical membrane

transporters (especially the cystic fibrosis transmembrane conductance regulator, CFTR), thereby promoting secretion of anions (Cl^- and HCO_3^-) by crypt cells and a decrease in absorption of Na^+ and Cl^- by absorptive cells (43). ST is a small monomeric toxin with multiple disulfide bonds, accounting for the heat stability of the toxin. Mature ST is either a 18-amino-acid peptide known as STp/ST1a, mostly present in porcine ETEC, or a 19- amino-acid peptide known as STh/ST1b, mostly present in human ETEC (44). ST is synthesized as precursor protein and translocated across the inner membrane via the Sec machinery (45), and subsequently through the outer membrane *via* TolC (46). Secreted ST molecules bind to the extracellular receptor domain of guanylyl cyclase C (GC-C) on the brush border of intestinal epithelium. These interactions activate the intracellular catalytic domain of GC-C, resulting in accumulation of intracellular cGMP and subsequent activation of cGMP-dependent protein kinase II, which leads to phosphorylation of the CFTR, and consequent chloride secretion (43). In addition, enhanced cGMP also inhibit activity of Na+/H+ exchangers (NHEs), resulting decreased fluid absorption (47, 48).

Since LT and ST toxins eventually induce excessive fluid loss with ensuing diarrhea, factors that influence secretion and/or delivery of these toxins ultimately affect virulence. Emerging evidence supports the concept that ETEC pathogenesis is more complicated than previously appreciated, and that optimal toxin delivery to target cells requires orchestrated involvement of multiple virulence factors. Interruption of any of the corresponding activities potentially abrogates toxin delivery and reduces ETEC virulence (33, 49). In order to investigate the genes that are involved in ETEC-host

interactions, we infected Caco-2 intestinal cells with ETEC and compared the transcriptome of cell associated ETEC with planktonic ETEC (29). This study identified multiple genes that are differentially regulated in cell associated ETEC, including increased transcription of genes that are involved in type 1 pili biogenesis (50), suggesting a potential role for these pili in ETEC-host interactions.

1.3 Type 1 pili

Type 1 pili are heteromeric fiber like structures, made up of a helical rod comprised of a major structural subunit polymer, FimA, joined to a tip consisting of three minor subunits, including FimF, FimG and FimH (51-53). These pili are encoded on the chromosome in the *fim* operon (54), and are expressed by both commensal and pathogenic *E. coli* (55). Type 1 pili are one of the most extensively studied chaperone-usher pathway (CUP) pili. The molecular detail of type 1 pili assembly has been described, outlining a paradigm for CUP pili biogenesis (53). Assembly of type 1 pili requires at least nine genes (Fig 1a), including *fimB* and *fimE* genes encoding two recombinases which control the pili biogenesis (56) by regulating expression of the major structural subunit encoding gene *fimA*. *fimC and fimD* genes encode the chaperone-usher export system which guides the folding and assembly of different subunits and translocates the pilus rod outside the cell as it polymerizes (Fig 1b). The *fimF*, *fimG* and *fimH* genes encode minor structural subunits which form the distal end of the pili where the FimH adhesin is positioned at the tip (52).

The invertible switch element that allows the promoter either in ‘ON phase’ orientation to drive transcription of *fimA* or in ‘OFF phase’ orientation to prevent *fimA* transcription

(57), permits bacteria to switch between piliated and nonpiliated states (56, 58). Of the two recombinases that control the switching between ON and OFF phases (59, 60), FimB recombinase inverts the switch element in both ON to OFF phase and OFF to ON phase orientations favoring pilus formation (61). On the other hand, FimE recombinase mediated inversion is biased towards ON to OFF phase orientation which stops pilus formation, resulting in nonpiliated bacteria (60, 61). Additionally, for efficient inversion of the switch element, both FimB and FimE require multiple accessory regulators, including the leucine-responsive regulatory protein (Lrp), the integration host factor (IHF), and the nucleoid-associated protein H-NS global regulators (61-63). Therefore, any factors that influence expression of FimB and FimE recombinases and/or the accessory regulators may affect type 1 pili formation. Consequently, type 1 pili expression is affected by different biological as well as environmental signals including temperature, culture conditions, and media composition (59, 60, 64). For instance, static broth culture favors type 1 pili expression, whereas growth of *E. coli* on agar favors nonpiliated bacteria (65). Similarly, *E. coli* growth at 28˚C cease type 1 pili expression yielding nonpiliated bacteria, whereas *E. coli* growth at 37˚C promotes type 1 pili expression (64). These complex sensory inputs may permit the bacteria to assess the appropriate niche for type 1 pili expression. For instance, 37˚C (body temperature) and neutral pH of human small intestine, which favor type 1 pili expression (61, 64), would allow *E. coli* to shift toward fimbriated state and promote their adhesion and colonization of small intestinal epithelia. Therefore, the multi-tier control of type 1 pili expression, by sensing both intrinsic and extrinsic factors, helps *E. coli* to express these adhesion molecules in an appropriate niche.

1.4 Type 1 pili in pathogen-host interactions

Bacterial attachment to the host is mediated by specific interactions between molecules on the surface of bacteria and the target receptors on the host tissues. *E. coli* possess a large number of CUP pili (66) that present the adhesin molecules to facilitate bacteria-host interactions. Although CUP pili utilize a general mechanism of biogenesis, each pilus operon comes with its native regulatory system as well as chaperone-usher system (66), suggesting that these operons might sense specific signal(s) for expression. For example, uropathogenic *E. coli* (UPEC) strains that cause acute pyelonephritis mostly express P-pili, another CUP pili which interacts with globoseries glycosphingolipids of human kidney cells (67, 68), while UPEC strains that cause cystitis mostly express type 1 pili which interact with mannosylated uroplakin of uroepithelium (69, 70). Additionally, in some cases both CUP pili, p pili and type 1 pili, might act in synergy for efficient pathogen-host interactions (71).

Type 1 pili have been reported as a major virulence factor for UPEC pathogenesis (72). These pili are important for multiple stages of UPEC pathogenesis, including attachment and invasion of uroepithelial cells (73, 74) and intracellular bacterial community formation (75). Type 1 pili are also a purported virulence factor for other pathogenic *E. coli,* including adherent-invasive *E. coli* (AIEC) that have been associated with Crohn's disease (CD) (76, 77). In CD patients, type 1 pili of AIEC recognize a highly mannosylated glycoproteins, carcinoembryonic antigen like cell adhesion molecule 6 (CEACAM6), which is present in excess on the intestinal epithelium of these patients (77-79). Therefore, pathogenic *E. coli* might utilize type 1 pili depending on the

environmental cues they sense in different niche, in facilitating specific bacteria-host interactions.

Since the FimH adhesin of type 1 pili is a mannose binding lectin, it can recognize a wide range of glycoconjugates carrying mannose structures, offering a broad binding range for interactions. Consequently, FimH has been shown to interact with myriad of host cells, including bladder and kidney epithelial cells (75), buccal cells (80), erythrocytes (81), mast cells (82), neutrophils (83), macrophages (84) as well as intestinal epithelial cells (79, 85). Additionally, FimH of type 1 pili bind to mannosylated proteins of Yeast causing agglutination (86). In order to facilitate these interactions, the FimH adhesin binds to a range of host molecules, including uroplakins (69), integrins (87), laminin (88), Tamm–Horsfall protein (89), CD48 (84), collagens (88), Toll Like Receptor 4 (TLR4) (90) and CEACAMs (85). Multiple studies have demonstrated that inhibition of FimH mediated interactions by anti-FimH antibodies (91) or by soluble D-mannose or mannosides (92, 93) as well as by a number of naturally occurring compounds containing terminal mannose residues (94-96) reduce pathogenesis, suggesting that these interactions are essential for the pathogenesis of many *E. coli* (78, 93, 97). Accordingly, type 1 pili are an important therapeutic target against pathogens that engage these pili in interactions with host.

1.5 Type 1 pili in ETEC

Although type 1 pili are important virulence factor for UPEC pathogenesis (72, 97) and the *fim* operons of UPEC and ETEC are virtually identical, their role in ETEC

pathogenesis has not been investigated. ETEC make type 1 pili that are distinct from the plasmid-encoded CFs (26, 98). The potential contribution of type 1 pili to ETEC adhesion was first suggested many years ago (98-100), and early attempts to use crude preparations of ETEC type 1 pili (then referred to as somatic fimbriae) in vaccines gave mixed results (99). While many recent studies identified multiple plasmid encoded novel virulence factors (35, 37, 49, 101), several studies suggested that chromosomally encoded type 1 pili may also be involved in ETEC pathogenesis (26, 98, 100). Nevertheless, recent demonstrations that type 1 pili encoding genes are up-regulated on cell contact (29) prompted a reexamination of their potential role in ETEC pathogenesis. Moreover, LT induces many changes in target epithelial cells, including mucin glycoprotein synthesis and secretion (37, 102), which might impact type 1 pili mediated pathogen-host interactions (103). Since type 1 pili encoding genes are part of the *E. coli* core genome, we speculate the presence of these genes in majority of the ETEC strains. Therefore, understanding the role of these pili might update our understanding of ETEC pathogenesis and determine whether type 1 pili mediated ETEC-host interactions are effective targets for broadly protective ETEC vaccines.

1.6 Hypotheses

Recently we observed significant induction of *fim* genes in ETEC H10407, a well characterized clinical isolate used in most of the ETEC pathogenesis studies, which were bound to intestinal epithelial cells. Additionally, deletion of the pilin subunit encoding *fimA* gene in H10407 eliminated mannose specific interactions, detected by the loss of yeast agglutination activity, a widely used assay for type 1 pili mediated

mannose specific interactions (Fig 2a). These observations suggested that type 1 pili are the only source of mannose specific interactions demonstrated by ETEC H10407. Moreover, *fimA* mutants were significantly less adherent than the wild type H10407 (WT) (Fig 2b) and methyl-α-D-mannose demonstrated significant inhibition of H10407 adhesion (not shown), comparable to *fimA* mutants, to Caco-2 intestinal cells. These observations and a report from earlier study (104) suggested that the mannose specific inhibition of ETEC adhesion is probably attributed to type 1 pili.

While ETEC make type 1 pili and the genes involved in the biogenesis of these pili are induced in contact with target epithelial cells, the contribution of these pili in ETEC pathogenesis has never been thoroughly investigated. Since earlier studies reported that intimate interaction with the host is essential for delivery of enterotoxins to the epithelial cells, type 1 pili mediated ETEC-host interactions might contribute significantly in the pathogenesis process. Therefore, we hypothesized that type 1 pili of ETEC may facilitate interactions with the host which promote toxin delivery to target epithelial cells. In order to test this hypothesis, we had following specific aims:

1) Investigate the role of type 1 pili in mediating adhesion of ETEC to the host intestinal epithelial cells and determine whether these structures are essential for the delivery of the ETEC enterotoxins.
2) Examine whether LT-induced changes in epithelial cells impact type 1 pili mediated ETEC-host interactions.
3) Investigate the contribution of type 1 pili in ETEC colonization to the intestinal epithelium.

CHAPTER ONE: FIGURES

a

fimB-fimE-fimA-fimI-fimC-fimD-fimF-fimG-fimH

b

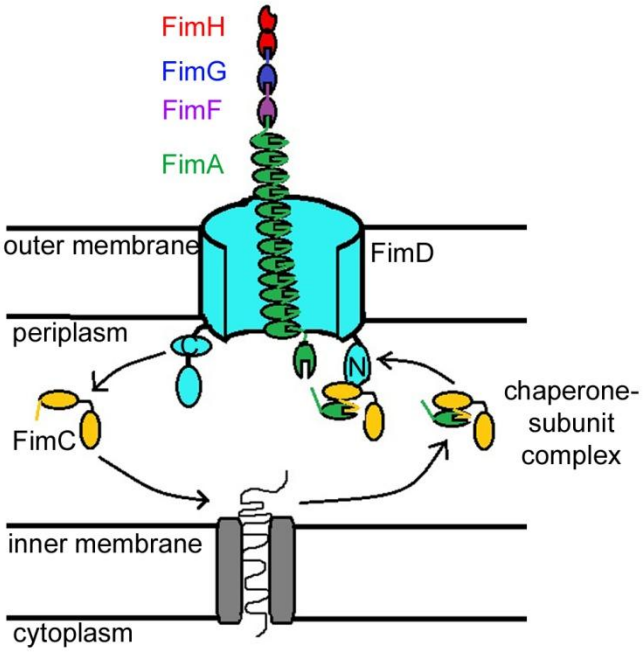

Figure 1. Biogenesis of type 1 pili.

a) Genes present in the *fim* operon. b) Schematic diagram of type 1 pili biogenesis (52, 53). Structural subunits are translocated to the periplasm via general secretory pathway, where the chaperone (FimC) guide their folding and transfer to the usher (FimD). Minor subunits, including FimF, FimG and FimH form the tip where FimH is located at the distal end, and the major structural subunit (FimA) polymerizes to form the pilus rod.

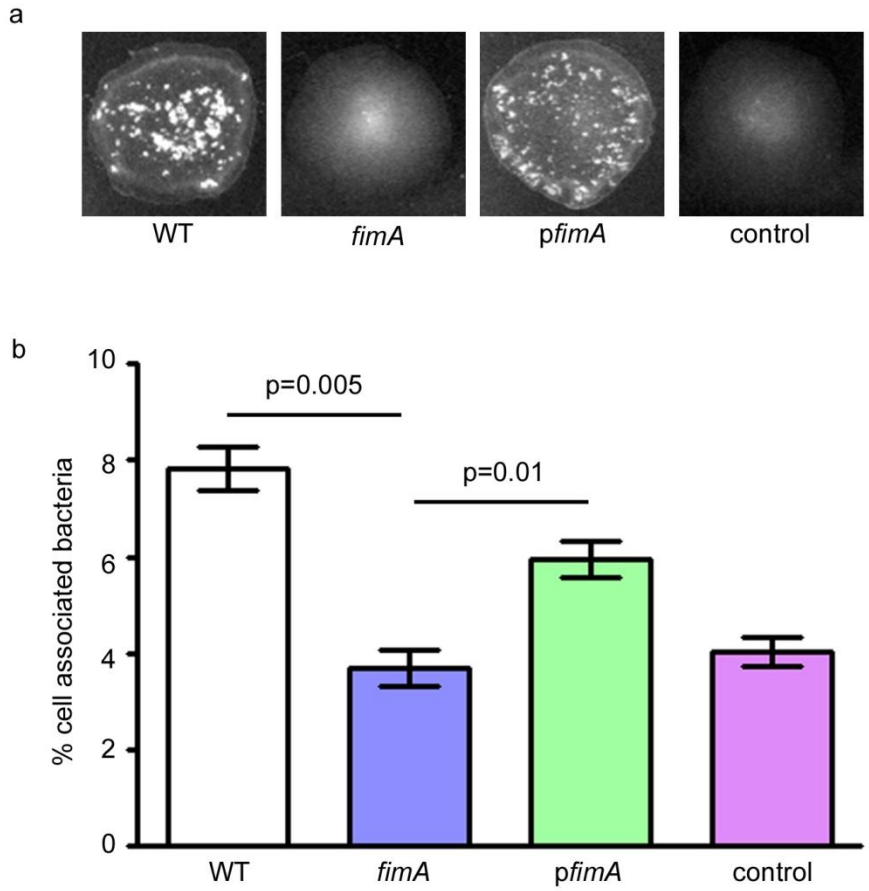

Figure 2. Type 1 pili mediated interactions of ETEC.

a) Yeast agglutination (YA) assays demonstrating type 1 pili specific interactions of ETEC with Baker's yeast. While deletion of *fimA* diminished YA activity, complementation of the mutant by episomal expression of FimA (p*fimA*) restored YA activity. b) Type 1 pili mediated adhesion to Caco-2 intestinal cells. The percentage of cell associated bacteria represents the portion of bacteria associated with the CIE at the end of 1 h relative to the inoculums. Bars represent mean values ± SEM of 5 replicates. P values were calculated by nonparametric Mann-Whitney test.

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CHAPTER TWO

Highly conserved type 1 pili promote enterotoxigenic *E. coli* pathogen-host interactions

ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC), defined by their elaboration of heat-labile (LT) and/or heat-stable (ST) enterotoxins, are a common cause of diarrheal illness in developing countries. Efficient delivery of these toxins requires ETEC to engage target host enterocytes. This engagement is accomplished using a variety of pathovar-specific and conserved *E. coli* adhesin molecules as well as plasmid encoded colonization factors. Some of these adhesins undergo significant transcriptional modulation as ETEC encounter intestinal epithelia, perhaps suggesting that they cooperatively facilitate interaction with the host. Among genes significantly upregulated on cell contact are those encoding type 1 pili. We therefore investigated the role played by these pili in facilitating ETEC adhesion, and toxin delivery to model intestinal epithelia. We demonstrate that type 1 pili, encoded in the *E. coli* core genome, play an essential role in ETEC virulence, acting in concert with plasmid-encoded pathovar specific colonization factor (CF) fimbriae to promote optimal bacterial adhesion to cultured intestinal epithelium (CIE) and to epithelial monolayers differentiated from human small intestinal stem cells. Type 1 pili are tipped with the FimH adhesin which recognizes mannose with stereochemical specificity. Thus, enhanced production of highly mannosylated proteins on intestinal epithelia promoted FimH-mediated ETEC adhesion, while conversely, interruption of FimH lectin-epithelial interactions with soluble mannose, anti-FimH antibodies or mutagenesis of *fimH* effectively blocked ETEC adhesion. Moreover, *fimH* mutants were significantly impaired in delivery of both heat-stable and heat-labile toxins to the target epithelial cells *in vitro, and* these mutants were substantially less virulent in rabbit ileal loop assays, a classical model of ETEC

pathogenesis. Collectively, our data suggest that these highly conserved pili play an essential role in virulence of these diverse pathogens.

AUTHOR SUMMARY

Enterotoxigenic *Escherichia coli* (ETEC) infections contribute substantially to death and morbidity due to diarrheal illness and are associated with serious sequelae including malnutrition, stunted growth, and intellectual impairment among young children in developing countries. Effective engagement of intestinal epithelial cells is essential for ETEC pathogenesis. Consequently, pathovar specific plasmid-encoded adhesin structures known as colonization factors (CFs) have been a principal target for vaccines. However, tremendous inter-strain variation in the carriage of gene clusters encoding different CFs and significant antigenic diversity of the CF adhesins has posed a challenge to vaccine development. In contrast, type 1 pili are encoded by the *fim* operon located in the chromosome of most ETEC strains and are highly conserved. While type 1 pili are known to play a critical role in virulence of extraintestinal pathogenic *E. coli,* the present studies represent the first detailed examination of the contribution of these pili in ETEC pathogenesis. Here we demonstrate that ETEC type 1 pili are essential for optimal interactions with intestinal epithelia and that they play a critical role in virulence. These data may inform additional approaches toward development of broadly protective vaccines for these pathogens of global importance.

INTRODUCTION

Among young children under five years of age in developing countries, diarrhea is a leading cause of morbidity and mortality. Enterotoxigenic *E. coli* (ETEC) is one of the most common causes of moderate to severe diarrheal illness and deaths due to diarrhea in young children and incidentally is also the leading bacterial cause of diarrhea [1]. These bacteria are also a leading cause of hospitalization due to severe diarrhea in adults in developing countries [2] and are perennially the predominant cause of diarrheal illness among travelers to the endemic regions [3, 4]. Additionally, ETEC infections contribute substantially to the burden of diarrheal illness associated with sequelae of malnutrition [5, 6], stunted growth [7] and impaired cognitive development [8]. The effects of ETEC infections also appear to be more critical in malnourished children [5]. Thus these pathogens contribute to a complex pattern of poverty, repeated enteric infections, environmental enteropathy [9], and developmental impairment .

ETEC are defined by the production of heat-labile (LT) and/or heat-stable (ST) enterotoxins [10], and virulence requires successful delivery of these toxins to cognate receptors on target intestinal epithelial cells. LT binds to cell surface GM1 gangliosides, and following cellular entry this toxin activates production of host cAMP; while ST peptides bind guanylate cyclase C, stimulating production of cGMP [11]. Resulting increases in intracellular concentrations of these cyclic nucleotides modulate ion channels on the surface of intestinal cells leading to net losses of sodium chloride and water into the intestinal lumen and ensuing acute watery diarrhea [11, 12]. In the classical paradigm of ETEC pathogenesis, these bacteria utilize pathovar-specific fimbrial or non-fimbrial adhesins, known as colonization factors or CFs [13] which allow

them to adhere and colonize the small intestine where toxin delivery occurs. However, emerging evidence would suggest that this paradigm is perhaps overly simplistic, and that there are several potential adhesins which effectively act in concert to promote ETEC engagement of the host [14].

E. coli encode a multitude of pili assembled by the **c**haperone/**u**sher **p**athway, termed CUP pili, which are important in virulence. CUP pili are tipped with specialized adhesins that recognize specific receptors with stereochemical specificity. CUP adhesins can determine both tissue tropism and the course of disease. For example, type 1 pili are encoded by the *fim* operon, and chomosomally encoded as part of the core *E. coli* genome [15, 16]. Type 1 pili are composite fibers comprised of a pilus rod, made up of FimA subunits arranged in a right handed helical cylinder [17]. The pilus rod is joined to a fibrillum structure tipped with the FimH adhesin that binds mannose with stereochemical specificity [16, 18-20]. FimH is critical for virulence in extraintestinal *E. coli*, as it has been well-established that FimH mediated adhesion enables uropathogenic *E. coli* (UPEC) colonization and invasion into bladder epithelial cells [21-23], as well as the formation of intracellular bacterial communities [24]. ETEC also encode pathovar-specific CUP pili (fimbriae) like CFA/I [25] that are encoded on virulence plasmids [26]. More than four decades ago, these ETEC-specific colonization factors were shown to contribute to development of diarrheal illness in humans [27], and consequently they have been the subject of intensive investigation and a major focus of ETEC vaccine development. Conversely, although early studies described possible type 1 pili expression by ETEC [28, 29], relatively little is known about the contribution of

these highly conserved structures to virulence. Our more recent observation that the expression of the *fim* operon is enhanced by pathogen-host cell contact [14], prompted a thorough investigation of the potential role of type 1 pili in ETEC pathogenesis reported here.

RESULTS

Type 1 pili promote ETEC binding to intestinal epithelia

Although a number of earlier studies of ETEC suggested that these pathogens make type 1 pili (also previously referred to as type 1 somatic pili or type 1 fimbriae) [29, 30], to date there has been no systematic examination of their involvement in ETEC virulence. We therefore first performed studies to confirm the production of type 1 pili by the prototypical ETEC H10407 strain. Type 1 pili tipped with the FimH adhesin were identified on the surface of strain H10407 by transmission electron microscopy after immunogold labeling using anti-FimH antibodies (Fig 1a). Similarly, using flow cytometry we verified production of type 1 pili in H10407, but not in the corresponding *fimH* mutant strain (Fig 1b). It has previously been shown that FimH is required to initiate the assembly of type 1 pili and thus *fimH* mutants are nonpiliated [31]. The expression of type 1 pili is under the transcriptional control of an invertible promoter element that governs phase OFF and phase ON populations [32, 33]. Interestingly, mutations that inactivate FimH such as the Q133K mutation, bias the *fim* promoter towards the phase OFF state [34]. Thus, compared to the wild type H10407 strain, or p*fimH* complemented mutants, isogenic *fimH* mutants or those complemented with pQ133K, which encodes FimH with a mutation in the mannose binding site [35], were nonpiliated and incapable

of yeast agglutination, a phenotypic assay for expression of type 1 pili [36] (Fig 1c) and only the wild type and *fimH* complemented mutants exhibited demonstrable type 1 pili expression detected by anti-type 1 pili antibodies in immunoblots (Fig 1d). Next, using polarized cultured intestinal epithelia (CIE) derived from the C2BBe1 clone of Caco-2 cells which produce apical brush borders with defined microvilli similar to human intestinal enterocytes [37], we demonstrated that production of FimH was required for effective adhesion. Mutants lacking *fimH* were significantly less adherent than wild type ETEC ($p<0.0001$) (Fig 1e,f), while episomal expression of the *fimH* gene (p*fimH*), but not the mutant *fimH* allele (p*Q133K*), restored adhesion. Additionally, H10407-*fimH*:Q133K, which contains the Q133K mutant allele of *fimH* in the chromosome, abrogated yeast agglutination activity and demonstrated significant decrease in adhesion to intestinal cells (data not shown). Similarly, mutants lacking the *fimA* gene encoding the major type 1 pili pilin subunit exhibited loss of functional type 1 pili in yeast agglutination assays, resulting in significant reduction of adhesion to the CIE (S1 Fig).

We found that methyl-□-D-mannose but not the methyl-□-D-galactose control sugar, inhibited FimH mediated yeast agglutination by ETEC (Fig 2a) and ETEC adhesion to epithelial cells (Fig 2c). Similarly, antibodies generated against the lectin domain of FimH (α-FimH), but not control antibodies, separated from pre-immune sera, inhibited FimH mediated yeast agglutination with wild type bacteria expressing type 1 pili (Fig 2b) and significantly inhibited ETEC adhesion to intestinal cells (Fig 2d). Collectively, these data support the idea that ETEC utilize type 1 pili to engage intestinal epithelia.

ETEC FimH adhesin binds to intestinal epithelia

We next examined the ability of the FimH tip adhesin to directly engage the intestinal epithelial surface. The purified lectin domain of FimH (FimHLD, 17 kD), representing amino acid residues 1-154 of mature FimH, bound to the apical surface of the CIE. In contrast, binding of FimHLD:Q133K, which lacks mannose binding activity, was markedly diminished (Fig 3a, b) as was binding of the wild type FimHLD protein in the presence of exogenous mannose (Fig 3b).

ETEC are noninvasive luminal pathogens thought to engage the microvilli at the apical surface of intestinal epithelial cells. Presumably, the FimH lectin can promote this engagement by interacting with mannosylated glycoconjugates on the glycocalyx covering the microvilli. Indeed, using transmission electron microscopy, we identified FimH by immunogold labeling at the ETEC-microvillus interface (Fig 3c).

Surface mannosylation enhances FimH-mediated ETEC-epithelial interaction

Because FimH interacts with mannosylated receptors on the epithelial surface, we examined the impact of enhanced glycoprotein mannosylation on ETEC pathogen host interactions. Following CIE treatment with kifunensine, an α-mannosidase class 1 enzyme inhibitor which enhances the display of high-mannose glycoproteins [38], we observed a significant increase in FimHLD binding (Fig 4a-b). Likewise, WT ETEC adhesion to the kifunensine treated CIE was enhanced relative to the untreated control CIE ($p<0.0001$) (Fig 4c,d). However, kifunensine treatment had no impact on adhesion

of the *fimH* mutant. These data further suggested that availability of mannosylated glycoproteins on the host cell surface promotes ETEC adhesion through FimH.

Type 1 pili act in concert with CFA/I fimbriae for optimal adhesion

Because ETEC H10407 expresses both type 1 pili and plasmid encoded CFA/I fimbriae specific to the ETEC pathovar, we investigated whether these CUP structures acted cooperatively in facilitating adhesion to intestinal epithelia. The CFA/I fimbriae are encoded by the *cfaABCE* operon in which *cfaE* encodes the CfaE tip adhesin [26, 39, 40]. Interestingly, mutations in either *fimH* or *cfaE* significantly reduced adherence of ETEC compared to wild type H10407, suggesting that both pili participate in ETEC adhesion (S2 Fig). Mutants lacking both the type 1 pili and the CFA/I tip adhesin genes (*fimH-cfaE*) demonstrated further reduction in adhesion compared to either of the single mutants (S2 Fig), supporting our hypothesis that structures encoded by these CUP pili act in concert.

Type 1 pili mediate adhesion to human small intestinal enteroids

While the CIE used in this study possess some features of normal intestinal epithelial cells, they are derived from metastatic colon cancer cells which fail to represent the diversity of cell types present in intestinal epithelium. Enteroids, derived from human intestinal stem cells collected from healthy volunteers [41-43], can recapitulate many aspects of normal physiology and preserve features of human intestinal epithelium. These include presentation of different cell types including enterocytes, goblet cells, Paneth cells, and endocrine cells [44-46]. Therefore, to further assess the contribution

of type 1 pili in ETEC adhesion to intestinal epithelia we established polarized intestinal epithelial monolayers derived from ileal specimens obtained from normal adult human subjects. In these enteroid-derived monolayers we were able to identify enterocytes with a defined brush border and distinct microvilli on the apical surface as well as goblet cells, and chromogranin A positive cells suggesting that they faithfully reproduce many features of surfaces normally presented to bacteria within the intestine (Fig. 5a-e).

Similar to our studies of CIE, we found that wild type ETEC adhered to the surface of stem cell derived polarized small intestinal (ileal) monolayers (Fig 6a,b) in close approximation with the microvillus surface (Fig 6c), and that mutation of *fimH* significantly attenuated adherence relative to the wild type parent strain (Fig 6d). Wild type bacteria adhered well to polarized monolayers of enteroid collected from multiple individuals, while the *fimH* mutant was persistently deficient in its ability to adhere to all target epithelia relative to the parent strain (Fig 6e). These studies suggested that type 1 pili of ETEC potentially play an important role in specifically directing bacterial interaction with the small intestine where release of toxins is thought to provoke the efflux of water and salt that lead to diarrhea.

Type 1 pili promotes optimal toxin delivery to intestinal cells *in vitro*

Close contact of ETEC with target epithelial cells is essential for efficient delivery of its enterotoxins [47, 48]. To investigate the impact of type 1 pili mediated ETEC-host interactions on toxin delivery, we measured the intracellular production of cAMP and cGMP, cyclic nucleotide second messenger markers for delivery of LT and ST,

respectively, in infected cells. cAMP and cGMP was significantly increased in target cells infected with WT H10407 relative to *fimH* or *fimA* mutants (Fig. 7a and b). Methyl-□-D-mannose significantly reduced levels of intracellular cGMP and inhibited cAMP activation in target cells infected with WT H10407 (Fig 7a and b). These effects were observed despite wild type levels of LT being produced by the mutants (Fig 7c). Together, these data suggested that type 1 pili are required for optimal delivery of both heat-labile and heat-stable enterotoxins to the target epithelial cells.

Type 1 pili are required for ETEC virulence

We next examined the contribution of type 1 pili to pathogenesis in the rabbit ileal loop model, a classical model of virulence for *V. cholerae* and ETEC [49]. Both *fimH* and *fimA* mutants were significantly less adherent than the WT H10407 to rabbit ileal intestinal epithelium (Fig 8 a,b). Likewise, while considerable fluid accumulation, a hallmark of ETEC virulence, was observed in loops infected with WT H10407 (Fig 8c) no demonstrable fluid accumulation was observed in control loops infected with *eltAB* mutants [47] which do not make LT toxin (S1 Table), or those containing only PBS, and we detected significantly less fluid accumulation in the loops infected with either the *fimH* or *fimA* mutants (Fig 8c), suggesting that type 1 pili mediated pathogen-host interactions contribute to ETEC virulence in this model.

Type 1 pili are highly conserved in diverse clinical ETEC isolates

To investigate the prevalence of functional type 1 pili expression in clinical ETEC isolates, we tested 174 geographically and phylogenetically disparate clinical isolates

including recently sequenced [50] strains using yeast agglutination assays. Overall, the majority (76%) of the clinical isolates demonstrated yeast agglutination activity indicative of preserved functional type 1 pili expression among ETEC. Importantly, we observed type 1 pili expression in isolates possessing each of the major colonization factors (CFs), and in 82% of isolates without any recognizable CFs (Table 1). Overall, these data suggest that functional type 1 pili are highly conserved in diverse ETEC clinical isolates.

Collectively data provided here demonstrate the first definitive evidence that type 1 pili are essential to the pathogenesis of these highly diverse pathogens. Similar to their well-established role in the pathogenesis of uropathogenic *E. coli* (UPEC) [51] where type 1 pili are required for interaction with bladder epithelia*,* these structures appear to be highly conserved in clinical isolates and are critical for ETEC adhesion and effective engagement of host intestinal epithelia that are ultimately required for efficient delivery of effector molecules including the known toxins*.*

DISCUSSION

Enterotoxigenic *E. coli* are a remarkably diverse group of pathogens that share plasmid-encoded effector molecules, namely heat-labile toxin (LT) and/or heat-stable toxins (ST). In effect, ETEC pathogenesis can be summarized by the virulence features that collectively facilitate the delivery of these toxins [52]. Successful engagement of the complex landscape presented by the intestinal mucosae that includes a secreted mucus layer as well as the glycocalyx, glycoconjugates on the apical surface of the epithelium

[53, 54], represents an essential step in ETEC virulence. Like other enteric pathogens, ETEC appear to employ a number of different adhesins (lectins) that recognize specific carbohydrate moieties on intestinal epithelia [55-58].

While most studies of ETEC adhesion, and consequently vaccine development, had previously focused on plasmid-encoded colonization factors, recent studies have suggested that bacterial adhesion, intestinal colonization, and toxin delivery ultimately represent very complex phenotypes involving the orchestrated deployment of a variety of pathovar specific plasmid encoded adhesins [14, 59, 60] as well as highly conserved chromosomally-encoded molecules [61] and other virulence factors including mucinases [62, 63] . Although a number of early studies had suggested that ETEC have the capacity to make type 1 pili [30, 64], their contribution to pathogenesis had not been comprehensively investigated.

Here, we demonstrate convincingly that most ETEC make type 1 pili, that these organisms utilize these highly conserved pili to engage the intestinal epithelium, and that these interactions are critical for effective delivery of both heat-labile and heat-stable toxins. Additionally, our data demonstrate that type 1 pili act in concert with the plasmid-encoded pathovar specific colonization factors to promote optimal interaction of H10407 with host intestinal epithelial surfaces. The precise interactions between type 1 pili and the host cell surface have not been thoroughly delineated, however, the data included here support the involvement of the minor pilin tip adhesin subunit (FimH) in mannose-dependent engagement of one or more host cell receptors [65-68].

Before the advent of recombinant techniques to construct isogenic deletion mutants, earlier investigations of ETEC H10407-P, a plasmid-cured strain of H10407 which lacks the large virulence plasmid encoding CFA/I colonization factor, Knutton *et. al.* suggested that type 1 pili mediated ETEC adhesion to human intestinal biopsies in a fashion that was inhibited by exogenous mannose [28, 29]. However, it was suggested that these pili were mediating adhesion to the basolateral surface of enterocytes rather than the apical side [28]. The data presented here overcome many of the technological limitations inherent in these earlier studies and demonstrate convincingly that type 1 pili mediate adhesion of ETEC to apical surface of small intestinal enterocytes, where LT and ST bind to surface GM-1 and guanylate cyclase C receptors, respectively.

While ETEC cause a tremendous burden of disease in low-middle income countries, and among travelers to these regions, at present, there is no suitable broadly protective vaccine to prevent infections caused by ETEC. This in part relates to substantial genetic and antigenic heterogeneity within the enterotoxigenic *Escherichia coli* pathovar. Most vaccines to date, in some form, have targeted the plasmid encoded colonization factor antigens. The remarkable heterogeneity of these antigens [69] and the fact that many ETEC [70, 71], more than half of isolates in some studies [72], do not make a recognizable colonization factor have prompted further investigation of these pathogens to define additional vaccine target antigens [73]. Our observation that ETEC isolates from a phylogenetically and geographically diverse collection of strains express functional type 1 pili can potentially inform alternative approaches to design of broadly protective immunogens.

In summary, the data presented here demonstrate that ETEC utilize type 1 pili for optimal engagement of the host intestinal epithelium and that these interactions accelerate toxin delivery to the target enterocytes essential for virulence. These studies provide an expanded view of ETEC molecular pathogenesis beyond the canonical paradigm envisioned more than 40 years ago, and potentially afford new avenues for the rational design of strategies to prevent the global burden of disease associated with these important pathogens.

METHODS

Mutagenesis and cloning

Isogenic *fimH* and *fimA* mutants (S1 Table) were constructed using lambda red mediated recombination as previously described [74].To construct *fimH* mutant, the primers jf101413.7 and jf101413.8 (S2 Table) were used to amplify the kanamycin resistance cassette from pKD4 plasmid with 60-bp tails corresponding to the DNA sequence immediately upstream and downstream of *fimH*. The resulting amplicon was then introduced into H10407 carrying the pKD46 helper plasmid for lambda red-mediated homologous recombination and mutants were selected on 50 μg/ml Kanamycin containing LB-agar plate, and tested for loss of *fimH* gene by PCR using primers jf120913.9 and jf120913.10. A complementation plasmid (p*fimH*) was constructed by amplifying the *fimH* gene with its native stop codon using primers jf120814.1 and jf120814.2 and cloning into pFLAG-CTC plasmid using infusion cloning kit (Clontech, Takara Bio, USA). Site-directed mutagenesis of *pfimH* with primers jf031814.1 and jf031814.2 was used to change the CAA codon corresponding to the

glutamine residue at position 133 of FimH to AAA codon corresponding to the lysine residue resulting in pQ133K (QuikChange, Stratagene, USA). Plasmids p*fimH* and p*Q133K* (S3 Table) were then introduced into the *fimH* mutants for complementation. All mutants were checked for motility, growth and secretion of known effector molecules including LT, EtpA and EatA.

To construct an expression plasmid for polyhistidine tagged FimH lectin domain (FimHLD), we amplified the N-terminal lectin region of *fimH* gene using primers jf042314.1 and jf042314.2. The amplicon was then cloned into pETDUET1 (In-Fusion, Clontech). Site directed mutagenesis was used to generate the mannose binding deficient FimHLD:Q133K as described above. The resulting plasmids, p*fimHLD* and p*fimHLD:Q133K*, were then introduced into BL21 (DE3) pLys strain for expression and purification of polyhistidine-tagged FimHLD and FimHLD:Q133K.

Expression and purification of recombinant protein

Overnight cultures of BL21 (DE3) pLys containing p*fimHLD* or p*fimHLD:Q133K* were diluted 1:100 into 2 liters of terrific broth supplemented with 100 μg/ml ampicillin and grown for 3 h at 37˚C to optical density at 600 nm (OD600) of ~0.7 then induced with 1 mM IPTG for 3 h at 30˚C. Cells were harvested, lysed and his-tagged proteins were purified from the bacterial lysates by nickel affinity chromatography using HisTrap HP column (GE healthcare bioscience, PA, USA). His-tagged FimHLD and FimHLD:Q133K were further purified by size exclusion column chromatography using HiLoad16/600 Superdex 200 pg column (GE).

Antibody generation and purification

Anti-FimH polyclonal rabbit antiserum was produced against the lectin domain of FimH as previously described [61]. Briefly, two New Zealand White rabbits were immunized (Rockland, USA) with recombinant polyhistidine-tagged FimHLD. Antibodies were separated from serum components using HiTrap columns prepacked with protein G Sepharose (GE). The resulting polyclonal antibodies were then pre-absorbed using lyophilized strain AAEC191-A [15], and affinity purification of antibody against FimHLD immobilized on nitrocellulose was performed as previously described [61, 75].

Bacteria and Cell culture

All experiments were carried out using prototypical ETEC strain H10407 or the isogenic mutants (S2 Table). Bactria were grown at type 1 pili inducing conditions by following static incubation for 24 h at 37˚C followed by subculturing at 1:100 for additional 24 h statically at 37˚C [34] in LB media supplemented with or without antibiotic, as appropriate, unless otherwise stated. C2BBe1 cells (ATCC Accession Number CRL-2102), a subclone of the Caco-2 cell colonic adenocarcinoma line, was used for generation of the cultured intestinal epithelium (CIE). In order to generate polarized monolayers which form an apical brush border, with microvilli morphologically comparable to that of the human intestinal epithelium [37], we seeded ~2 x10^5 C2BBe1 cells onto Transwell filters (0.4μM polystyrene membrane, 6.5mm diameter insert) in DMEM media supplemented with 10% FBS and 10 μg/ml human transferrin (Lonza, MD, USA) and grew at 37˚C with 5% CO_2 for three weeks for CIE (polarized monolayer culture). Media were changed every 2-3 days. Formation of microvilli was verified by

transmission electron microscopy. Caco-2 cells (ATCC) were cultured in MEM media supplemented with 20% FBS. T-84 (ATCC) cells were cultured in DMEM/F12 (1:1) media supplemented with 5% FBS.

Flow cytometry

Strains grown under type 1 pili inducing conditions were processed for flow cytometric analysis. For surface staining, bacterial pellets were washed once with PBS, fixed in 2% paraformaldehyde for 15 min and then incubated with 1%BSA in PBS for 30 min at RT. Bacteria were then incubated with primary antibody (α-FimH) at RT for 45 min. After washing with PBS, secondary antibody staining was performed at RT in the dark with species-specific antibodies conjugated with AlexaFluor 546 for an additional 45 min. Bacteria were then washed with PBS and resuspended in 100 µl of PBS for acquisition by flow cytometry (FACSCalibur, BD Biosciences). A minimum of 50,000 organism counts were acquired (CellQuest software, Becton Dickinson), and subjected to subsequent analysis (FlowJo, v7.6.3).

Type 1 pili extract preparation and detection:

Type 1 pili were extracted by following a previously described method with some modifications [76]. Briefly, bacteria grown in type 1 pili inducing conditions were harvested, re-suspended in 1 ml of 1 mM Tris-HCl (pH 8.0) and incubated at 65°C for 1 h with occasional vortexing and pelleted by centrifugation (15,000 × *g* for 5 min). The supernatant was then transferred to another tube, and an aliquot was precipitated in salt (300 mM NaCl and 100 mM $MgCl_2$) by incubating overnight at 4°C, followed by

centrifugation at 20,000 × *g* for 10 min at 4°C. The protein pellet was re-suspended in 1 mM Tris-HCl. Fimbrial extracts were separated by sodium dodecyl sulfate (SDS)-15% polyacrylamide gel electrophoresis minigels (Bio-Rad). Proteins were either stained with Coomassie brilliant blue or transferred to nitrocellulose membranes (Bio-Rad) using a Mini Trans-Blot electrophoretic cell (Bio-Rad) for 60 min at 100 V. The membrane was blocked with 5% milk-PBS supplemented with 0.05% Tween 20 (Pierce). Incubations with primary (1:5,000) and secondary (1:5,000) antibodies were carried out for 1 h at RT. Chemiluminescent substrate (Clarity Western ECL substrate, Bio-Rad) was used for detection.

Yeast agglutination assay

Yeast agglutination as a phenotypic test for the production of type 1 pili [36], was performed using the following conditions: strains to be tested were grown in 1 ml Luria broth at type 1 pili inducing conditions, harvested and adjusted to OD600 of 1.0 in PBS. Agglutination was performed on glass slides by mixing 20 µl bacteria with an equal volume of bakers' yeast suspension adjusted to OD600 of 1 in PBS.

Adhesion assays

For adhesion assays, bacteria to be tested were grown at type 1 pili inducing conditions and diluted at 1:10 in pre-warmed cell culture media prior to infection. Serial dilutions of each inoculum were plated on Luria agar for CFU count. Infected CIE were incubated at 37°C and 5% CO_2 for 1 h, washed with pre-warmed tissue culture medium 3 times with gentle shaking (100 rpm) for 1 min each. The CIE were then either lysed in 0.1% Triton

X-100 for 5 min, and the cell associated bacteria were recovered by plating lysates onto Luria agar, or processed for fluorescence microscopy for enumeration of bacteria attached to the cells.

Fluorescence microscopy

To investigate the binding of FimH in the context of intestinal epithelial cells, 100 µl of 50 µg/ml biotinylated FimHLD or FimHLD:Q133K was incubated with CIE. After an hour of incubation at 37˚C unbound FimHLD were washed off with tissue culture media, the cells were fixed with 2% paraformaldehyde for 30 min at room temperature (RT), washed twice with PBS, and blocked with 1% BSA-PBS for another 30 min at RT. Streptavidin coated Qdot 594 was then used at 1:100 dilution in 1% BSA-PBS to detect biotinylated FimHLD bound to the cell surface. For detection of ETEC adhesion, infected CIEs were incubated with anti-O78 antibody followed by fluorescent labeled secondary. Cell membranes and nuclei were stained as previously described (CellMask, red for membrane and DAPI for nuclei; Invitrogen) [61]. Images were acquired on a Zeiss LSM510 confocal microscope, and files were converted to TIFF image using ImageJ (v1.45). Signals were quantified using Volocity software (version 6.2; PerkinElmer, Inc.).

Transmission immunoelectron microscopy

For localization of FimH on intestinal epithelium, ETEC infected CIE were fixed in 2% paraformaldehyde/0.02% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM PIPES/0.5 mM $MgCl_2$, pH 7.2 for 1 h at 4°C. Samples were washed with PIPES

buffer, blocked with 5% FBS/5% NGS for 20 min and subsequently incubated with rabbit anti-FimH antibody for 1 h, followed by secondary goat anti-rabbit antibody conjugated to 18 nm colloidal gold for 1 h. Samples were washed in buffer and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 h. Samples were then rinsed extensively in dH_2O prior to en bloc staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h. Following several rinses in dH_2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) equipped with an AMT 8 megapixel digital camera (Advanced Microscopy Techniques, Woburn, MA).

Growth and differentiation of human small intestinal epithelial cells

Enteroids were grown as previously described in detail [45] from purified intestinal cell lines, maintained at the Washington University Digestive Diseases Research Core Center BioSpecimens Core under a protocol approved by the Institutional Review Board. Briefly, cells were thawed and re-suspended in Matrigel (BD Biosciences, San Jose, CA, 15 μL/well in 24 well plates), and incubated at 37°C with a 1:1 mixture of L-WRN conditioned media (CM) and primary culture media (Advanced DEM/F12, Invitrogen) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 10 μM Y-27632 (ROCK inhibitor; Tocris Bioscience, R and D Systems, Minneapolis, MN), and 10 μM SB 431542 (transforming

growth factor-ß type 1 receptor inhibitor; Tocris Bioscience, R and D Systems). For polarization, cells were added to Transwell filters (Corning) and incubated in differentiation media (1:20 mixture of L-WRN CM and primary culture media) lacking SB 431542. Cells were grown to confluence for 3 days in differentiation media before use.

Histological analyses of eneteroid-derived human intestinal epithelial monolayers

For histological analysis (Day 3), enteroids grown on transwell were fixed in 3.7% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min, washed once with PBS and processed for paraffin embedding. Transverse sections (5 µm) were stained with hematoxylin and eosin (visualized with a Zeiss Axioskop 2 MOT microscope fitted with a CRI Nuance FX multispectral imaging system, Cambridge Research and Instrumentation), or used for immunostaining. For immunostaining, sections were de-paraffinized and hydrated, boiled in Unmasking Solution (Vector Laboratories, Burlingame, CA) for 25 min, rinsed in PBS, blocked in 1% bovine serum albumin/0.1% Triton-X100 for 30 min and incubated with primary antibody at 4°C overnight. Primary antibodies included rabbit anti- ChgA (1:100, Abcam, Cambridge, MA), rabbit anti-Muc2 (1:100, Santa Cruz Biotechnology, Inc., Dallas, Texas), mouse monoclonal anti-Villin 1 (1:100; Santa Cruz Biotechnology, Inc.). Slides were rinsed 3 times with PBS and incubated for 60 min at RT with species specific secondary antibodies (1:200; Invitrogen) conjugated to AlexaFluor488 or AlexaFluor546. For detection of bacteria, enteroids (grown for 3 days) were infected for 1 h, washed 3 times with pre-warmed media, fixed in 3.7% paraformaldehyde and either processed for fluorescent microscopy or embedded in paraffin. For the detection of tight junction

formation, antibody against zonula occludens-1 (anti-ZO-1, mouse monoclonal, Invitrogen) was used at 1:100 dilutions followed by anti-mouse secondary antibodies conjugated to AlexaFluor488 (1:200, Invitrogen). Paraffin embedded sections were deparaffinized and processed for immunostaining with anti-O78 antibody (1:200) followed by secondary antibodies (1:200; Invitrogen) conjugated to AlexaFluor 546 for the detection of cell associated bacteria. UEA-1 lectin conjugated to FITC (1:100, Sigma) was applied during secondary antibody incubation. Slides were washed 3 times in PBS and stained with DAPI (Molecular Probe) to visualize nuclei and cellmask red (1:2000, Invitrogen) to visualize plasma membrane and mounted with ProlongGold antifade reagent (Molecular Probes) for confocal microscopy (Nikon ECLIPSE Ti confocal microscope equipped with NIS-Elements imaging software). To examine ETEC association with microvilli of polarized primary human small intestinal epithelial cells, infected monolayers were processed for transmission electron microscopy following protocol mentioned above.

Toxin secretion assay

LT toxin secretion by different strains was measured using previously established GM1-ELISA method [77]. Briefly, ELISA plates (Costar, Corning, NY) were coated with 100 µl/well of 1 µg/ml monosialoganglioside GM1 (Sigma, G-7641), and incubated overnight at RT. After blocking with 2% BSA in PBS-Tween, 100 µl of clarified culture supernatant from overnight Luria broth cultures of bacteria was added to wells in triplicate and incubated for 1 h at 37˚C. The wells were then washed and incubated with 100 µl of a 1:5000 dilution of rabbit anti-LTB polyclonal antisera for 1 h. Plates were again washed

and then incubated with 100 µl of a 1:5000 dilution of anti-rabbit secondary conjugated with HRP for another hour. Finally plates were developed with 100 µL per well of TMB-H_2O_2 (KPL) substrates and read immediately at 630nm for kinetic measurement (Eon, BioTek instruments, VT, USA).

Cyclic nucleotide assays

Caco-2 cell monolayers grown in 96-well plates were infected with WT or mutant strains and used to determine alterations in cAMP. After 2 h of infection wells were washed with pre-warmed tissue culture media, incubated for an additional 2.5 h, cells lysed in sample buffer and intracellular cAMP concentrations were then determined by ELISA (cAMP Direct EIA, Arbor Assays, MI, USA). Similarly, T-84 cell monolayers grown in 96-well plates were used to determine cGMP concentrations following infection. 1.5 h after infection, wells were washed 3 times with pre-warmed tissue culture media, incubated for an additional 3 h, lysed sample buffer, and intracellular cGMP determined (cGMP Direct EIA, Arbor Assays, MI, USA).

Rabbit ileal loop assays

The rabbit ileal loop (RIL) assay was performed as previously described [49, 78]. Briefly, bacteria were grown overnight in Luria broth at 37°C, at type 1 pili indicung conditions, then washed with PBS, and diluted yield to a target inoculum of ~1X10^6 CFU/ml/loop. Adult albino rabbits (New Zealand strain) weighing 1.5–2.0 kg were fasted for 24 h and allowed only water. Following induction of anesthesia with intravenous sodium pentobarbital (0.5 mL/kg body mass), the abdominal cavity was entered, the intestine

exposed and the ileum localized. Here, loops of 5 cm in length were created with surgical ligatures, with 2 cm intervals between each loop to yield 5-6 loops per rabbit. Loops were then inoculated with either wild type H10407 as a positive control or mutant strains. Animals were euthanized ~18 h post infection with sodium pentobarbital, the intestine exposed and the ileocaecal region was then removed. The volume of fluid accumulation determined per unit length of gut within each segment, after which tissue specimens from the corresponding segment were collected, fixed in formalin (10%), paraffin-embedded and subsequently processed for examination of villous associated bacteria following protocol mentioned above.

Ethics statement

Rabbit ileal loop protocol conducted at icddr,b was reviewed and approved by the institution's Animal Experimentation Ethics Committee (AEEC). Experimental procedures were carried out by veterinarians at the animal facility in accordance with relevant guidelines. The rules and guidelines followed at icddr,b for animal care and use adhere to the recommendations, with some modifications, stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH). These guidelines and the subsequent modifications were approved by the icddr,b Board of Trustees.

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CHAPTER TWO: REFERENCES

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CHAPTER TWO: FIGURES

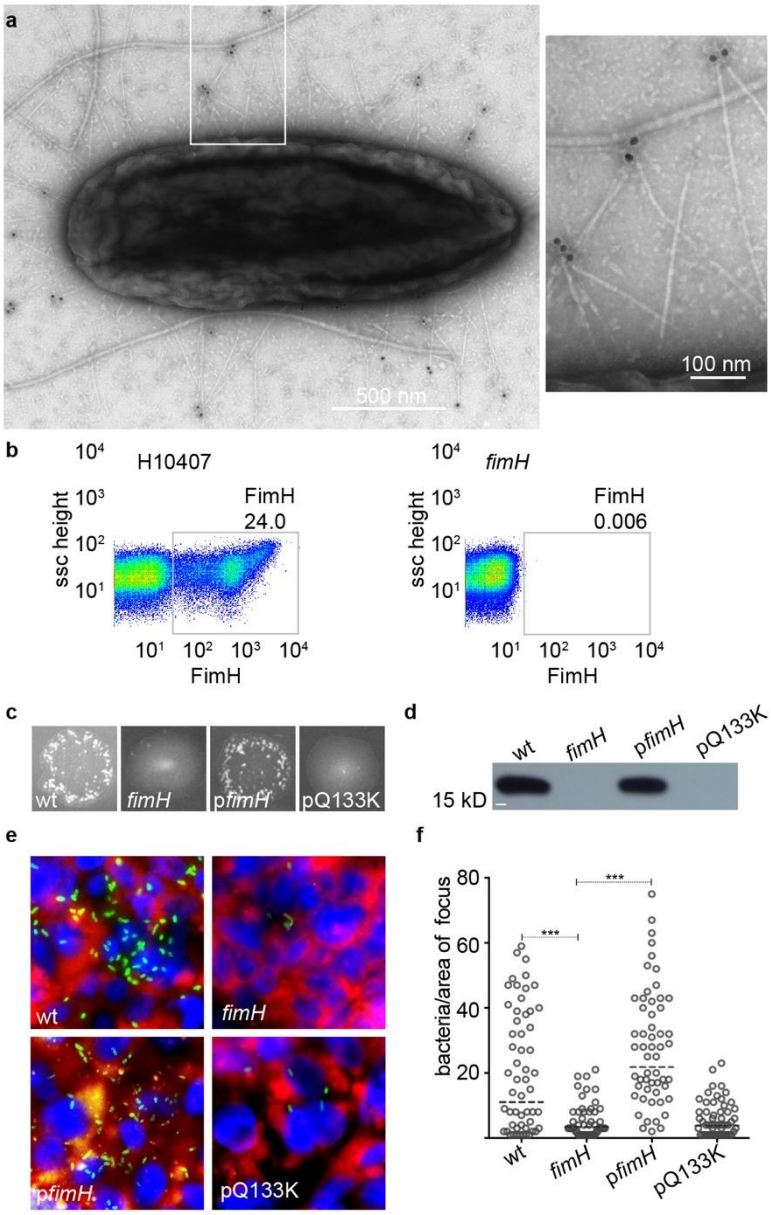


Figure 1. Type 1 pili expression promotes optimal adhesion of ETEC to intestinal epithelia. a. Transmission electron micrograph of ETEC H10407 expressing type 1 pili. The FimH tip adhesin was detected using α-FimH antibody and gold secondary antibody conjugate. **b**. Flow cytometric analysis of type 1 pili expression by ETEC

H10407 and *fimH* mutants. **c.** Assessment of type 1 pili function using yeast agglutination assays. Negative yeast agglutination reflected the loss of type 1 pili activity. **d.** FimA immunoblot of type 1 pili extracts from static culture of WT ETEC, *fimH* mutants and mutants complemented with wild type *fimH* gene (p*fimH*) or mutant *fimH* gene (pQ133K). **e.** Confocal microscopic images showing adhesion of WT ETEC, *fimH* mutants or complemented mutants to polarized cultured intestinal epithelia. Bacteria (anti-O78, green), cell membrane (CellMask, red), nuclei (DAPI, blue). **f.** Quantitative analysis was done by counting number of bacteria per focus area. Horizontal dashed lines represent geometric means of 3 combined individual experiments. P values were calculated by nonparametric Mann-Whitney test. *** indicates $p<0.0001$.

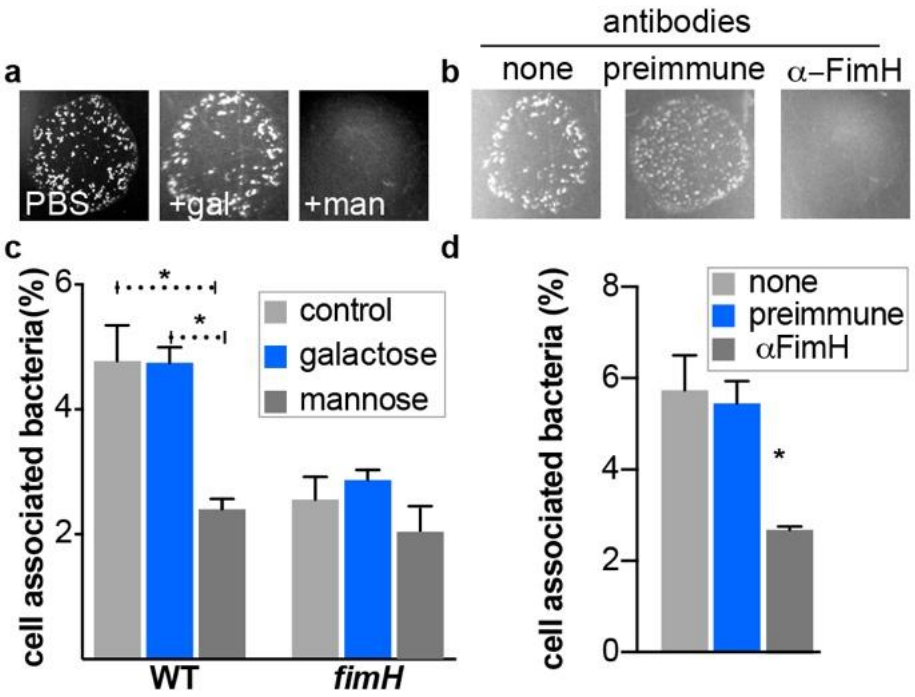


Figure 2. Inhibition of type 1 pili mediated interaction impairs ETEC adhesion.

Yeast agglutination assay of WT ETEC (**a)** is inhibited by methyl-α-D-mannose but not equimolar concentrations of methyl-α-D-galactose control sugar. **b.** Anti-FimH antibodies, generated against the lectin domain of FimH, but not preimmune sera inhibit yeast agglutination. **c.** *In vitro* adhesion of WT ETEC or *fimH* mutants in the absence or presence of methyl-α-D-galactose or methyl-α-D-mannose. **d**. WT ETEC adhesion is inhibited by anti-FimH antibodies. The percentage of cell associated bacteria represents the proportion of bacteria associated with the CIE at the end of 1 h relative to the inoculum. Bars represent mean vaules + SEM (n=5). P values were calculated by nonparametric Mann-Whitney test. * indicates $p<0.05$.

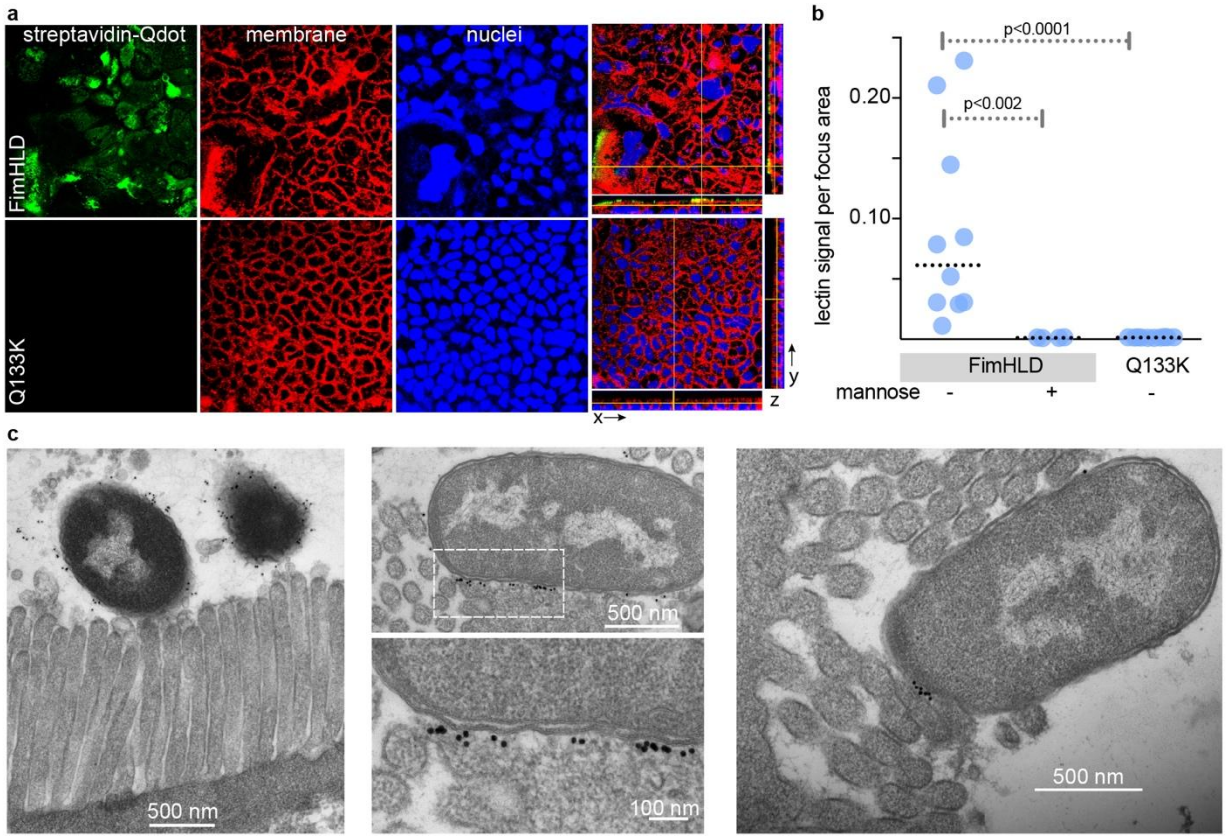


Figure 3. FimH adhesin of ETEC interacts with intestinal epithelial cells. a. Confocal microscopy images show binding of the biotinylated FimH lectin domain (FimHLD) or FimHLD:Q133K to the apical surface of cultured intestinal epithelium (CIE). Biotinylated FimHLD was detected with streptavidin-conjugated fluorescent nanocrystals (Qdot, green); plasma membranes were stained with CellMask (red) and nuclei with DAPI (blue). Image at right shows three dimensional reconstruction of z stacks of CIE following interaction with FimHLD or the mutant protein. **b.** Quantitative analysis of FimHLD binding to CIE represented in panels **a** using Volocity three-dimensional (3D) image analysis software (version 6.2; PerkinElmer, Inc.). P value was calculated using nonparametric Mann-Whitney testing. **c.** Immunoelectron microscopy

images of CIE infected with ETEC H10407. Left panel, microvilli structure at the apical surface of the CIE; right panels show immunogold labeling of FimH localized to the ETEC-host interacting surface.

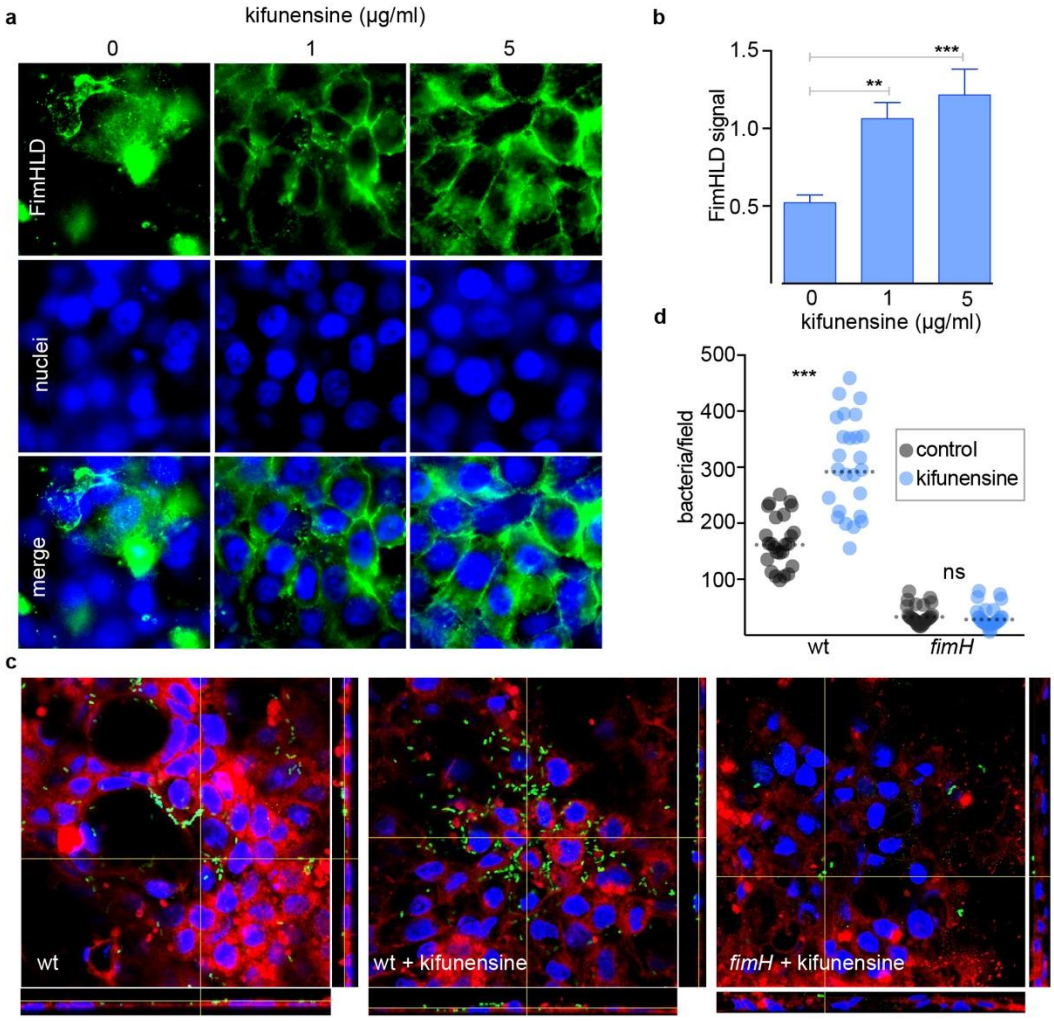


Figure 4. Enhanced presentation of mannosylated glycoproteins increases FimH binding and ETEC adhesion. a. Confocal microscopy images detecting FimHLD binding to the kifunensine treated CIE. Biotinylated FimHLD was detected with streptavidin-conjugated fluorescent nanocrystals (Qdot, green) and nuclei with DAPI (blue). **b.** Quantitative analysis of FimHLD binding to CIE represented in panels **a** using Volocity three-dimensional (3D) image analysis software (version 6.2; PerkinElmer, Inc.). **c.** Confocal microscopic images showing ETEC adhesion to kifunensine treated

CIE. The CIE grown on trans-well filters were treated with kifunensine and infected with WT ETEC or *fimH* mutants. One hour post infection wells were processed for microscopic examination. Bacteria (green), cell membrane (red), DAPI (blue). **d.** Quantitative analysis was done by counting number of bacteria present per focus area. Horizontal dashed line represent mean of 2 individual experiments. P values were calculated by nonparametric Mann-Whitney test. *** indicates $p<0.0001$.

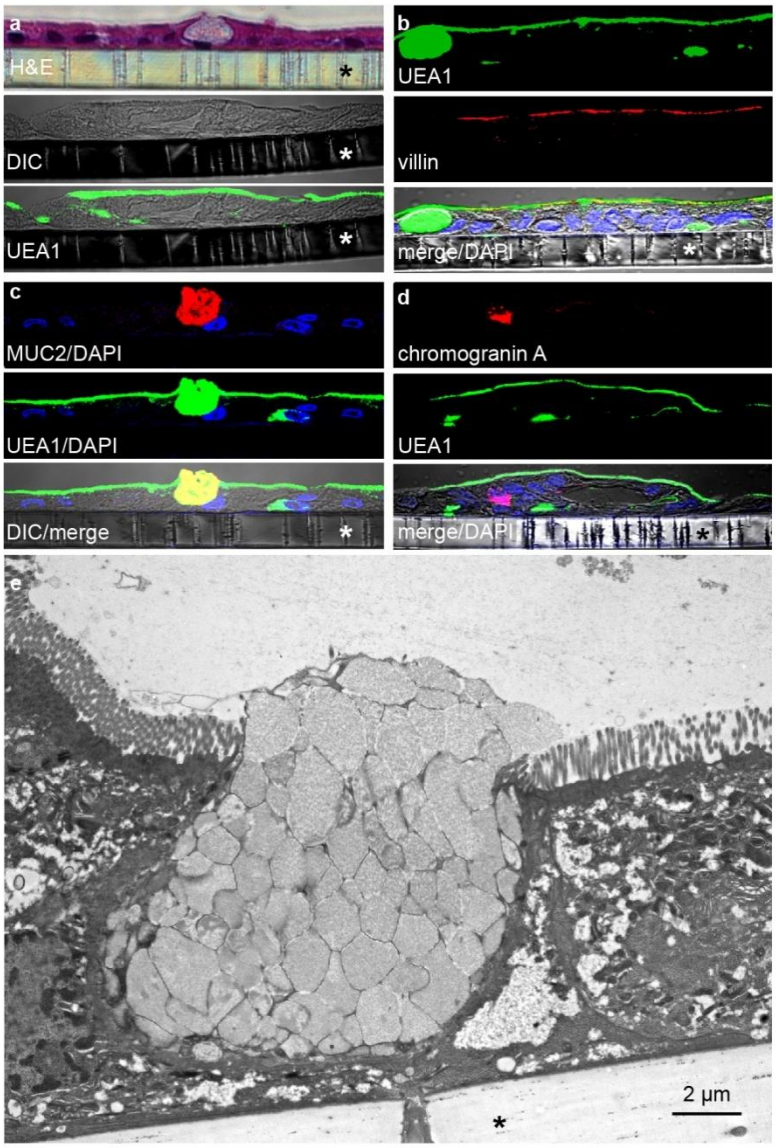


Figure 5. Properties of small intestinal enteroid cultures. a. Hematoxylin and eosin staining, DIC, and UEA1 lectin immunofluorescence confocal microscopic images showing formation of continuous monolayers. The apical surface is detected in the bottom image with FITC conjugated UEA1 lectin. **b.** UEA1 and anti-villin 1 antibody immunofluorescence in Laser Scanning Confocal Microscopy (LSCM) images from sections of polarized small intestinal enteroid monolayers. Nuclei (DAPI) are shown in

blue. **c.** LSCM of sections showing MUC2 immunofluorescence (red) in probable goblet cell and co-localization with UEA1 in the merged image. **d.** Chromogranin A positive cells (red). **e.** Transmission electron microscopy (3000x) of polarized small intestinal enteroid monolayer sections showing a goblet cell flanked by enterocytes with distinct microvilli on the apical surface. Transwell filters in sections are indicated by *.

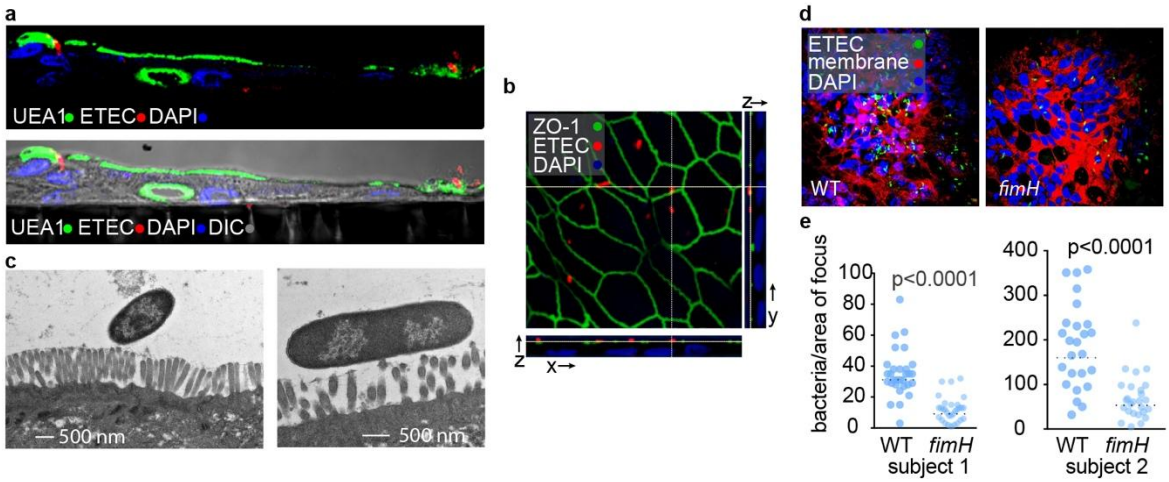


Figure 6. Type 1 pili are required for optimum adhesion to human small intestinal enteriods. a. Representative laser scanning confocal microscopy (LSCM) images of sections prepared from polarized enteroid small intestinal monolayers infected with WT H10407 (red, anti-O78). Surface staining with the UEA1 lectin is shown in green. Nuclei are stained in blue (DAPI). **b.** Three dimensional reconstruction of LSCM z stacks of ETEC H10407 infected polarized monolayer of enteroids showing the distribution of zonula occludens-1 (ZO-1, green) at the apical surface. Bacteria were visualized with anti-O78 (red) and nuclei are stained with DAPI (blue). **c.** Transmission electron microscopy images of ETEC H10407 adhering to microvilli on the surface of small intestinal enteroids (magnification 7500x and 15000x for left and right images respectively). **d.** LCSM images of wild type (wt) versus *fimH* mutant bacteria adherent to the surface of small intestinal enteroids Bacteria were visualized with anti-O78 (green) and cell membranes with CellMask (red), nuclei (DAPI, blue). **e.** Quantitative analysis of ETEC adhesion to enteroids represented in panel **d**. Each dot plot represents adhesion data obtained using ileal enteroids derived from two individual subjects. Horizontal lines

represent geometric means of data combined from 2 independent experiments. P values were calculated by nonparametric Mann-Whitney testing.

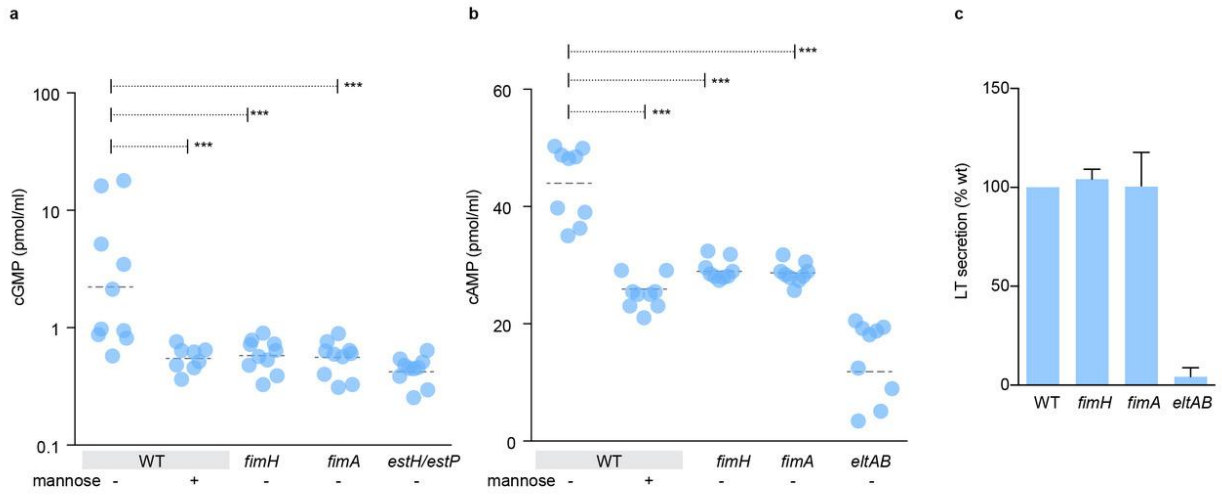


Figure 7. Type 1 pili mediated interactions enhance toxin delivery.

a. Quantification of intracellular cGMP in infected cells. Cells were infected with WT ETEC in the absence or presence mannose sugar or with *fimH* or *fimA* mutants. Cells infected with *STp/STh,* ST mutants, represent basal level of cGMP in cells. **b.** Quantification of the amount of intracellular cAMP in infected cells. Cells were infected with WT ETEC in the absence or presence mannose sugar or with *fimH* or *fimA* mutants. Cells infected with *eltAB,* LT mutants, represent basal level of cAMP in cells. **c.** LT secretion by different mutants. Each bar represent mean with SEM (error bar) of 2 experiments consisting of 5 replicates per experiment for each strain. All P values were calculated by nonparametric Mann-Whitney test. *** $p<0.0001$.

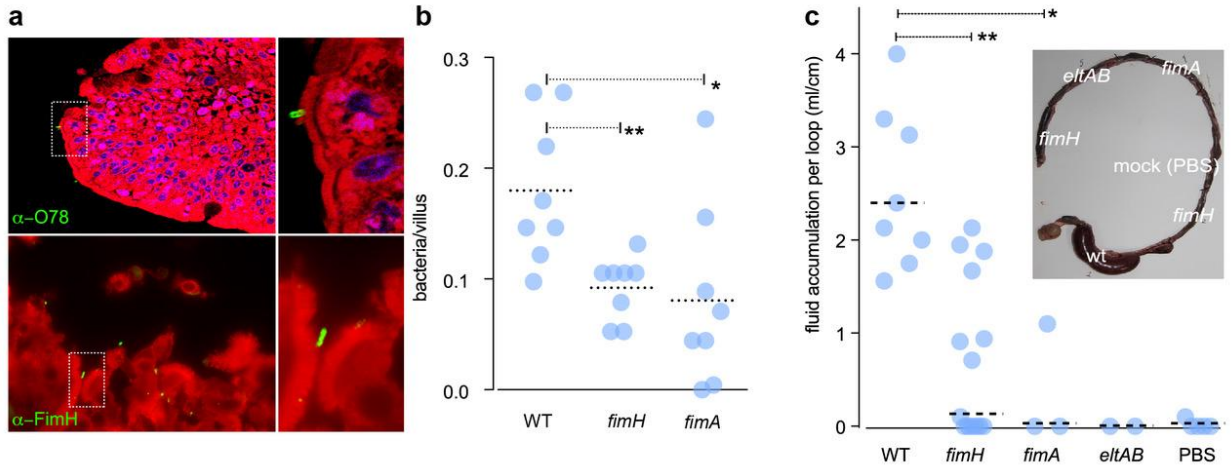


Figure 8. Type 1 pili are required for virulence in the rabbit ileal loop assay.

Type 1 pili are required for optimal bacterial engagement of rabbit intestinal epithelia. **a.** sections of rabbit ileum stained with anti-O78 (bacteria top, green) or anti-FimH (bottom). Nuclei are stained with DAPI (blue) and membranes are stained with CellMask (red). **b.** bacteria adherent to the ileal mucosal surface following infection with wild type ETEC H10407 or *fimH* and *fimA* mutants. **c.** Type 1 pili are required for toxicity in the rabbit ileal loop assay. Shown in the graph is the amount of fluid accumulation in each loop infected with WT or *fimH* or *fimA* mutants 18 h post inoculation. Loops infected with *eltAB* mutants or mock infected (PBS) were used as controls. Date represent summary of experiments from different rabbits (n=7). Inset shows image of infected ileal loop of one representative experiment.

CHAPTER TWO: TABLE

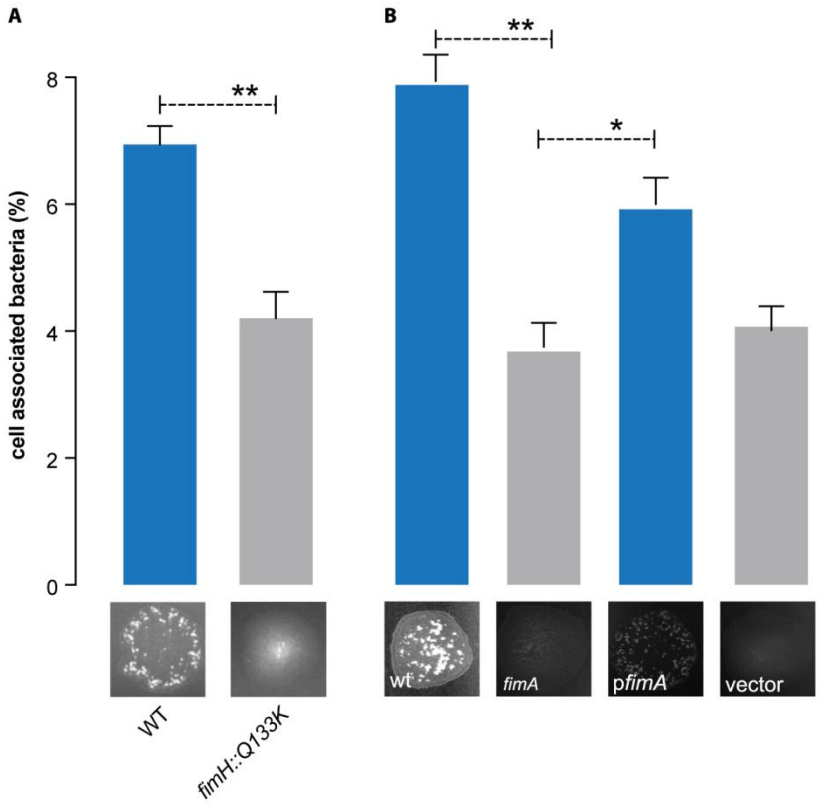
Table 1: Relationship of functional type 1 pili expresion to colonization factors

CF designation(s)	CF expression		Type 1 pili expression	
	number	%	number	%
CFA/I, CS21	28	16	26	93
CS1, CS3, CS21	15	8.6	2	13
CS7	14	8	14	100
CS2, CS3, CS21	11	6.3	10	91
CS5, CS6	9	5.2	9	100
CFA/I	7	4	7	100
CS1, CS3	6	3.4	2	33
CS21	6	3.4	2	33
CS17	5	2.9	5	100
CS20, CS21	5	2.9	5	100
CS4, CS6	5	2.9	1	20
CS2, CS3	4	2.3	2	50
CS14	3	1.7	2	67
CS6, CS21	3	1.7	3	100
CS1	2	1.1	2	100
CS1, CS3, CS20,	2	1.1	0	0

CS21				
CS6	2	1.1	2	100
CS6, CS20, CS21	2	1.1	2	100
CS6, CS8	2	1.1	2	100
CFA/I, CS20, CS21	1	0.6	1	100
CS1, CS21	1	0.6	0	0
CS12, CS20	1	0.6	1	100
CS12, CS20, CS21	1	0.6	1	100
CS19	1	0.6	1	100
CS2, CS3, CS20	1	0.6	1	100
CS2, CS3, CS20, CS21	1	0.6	0	0
CS5	1	0.6	1	100
CS5, CS6, CS21	1	0.6	1	100
Undetected	34	19.5	28	82
total + isolates	140*	80	133*	76

*of n=174 total isolates queried

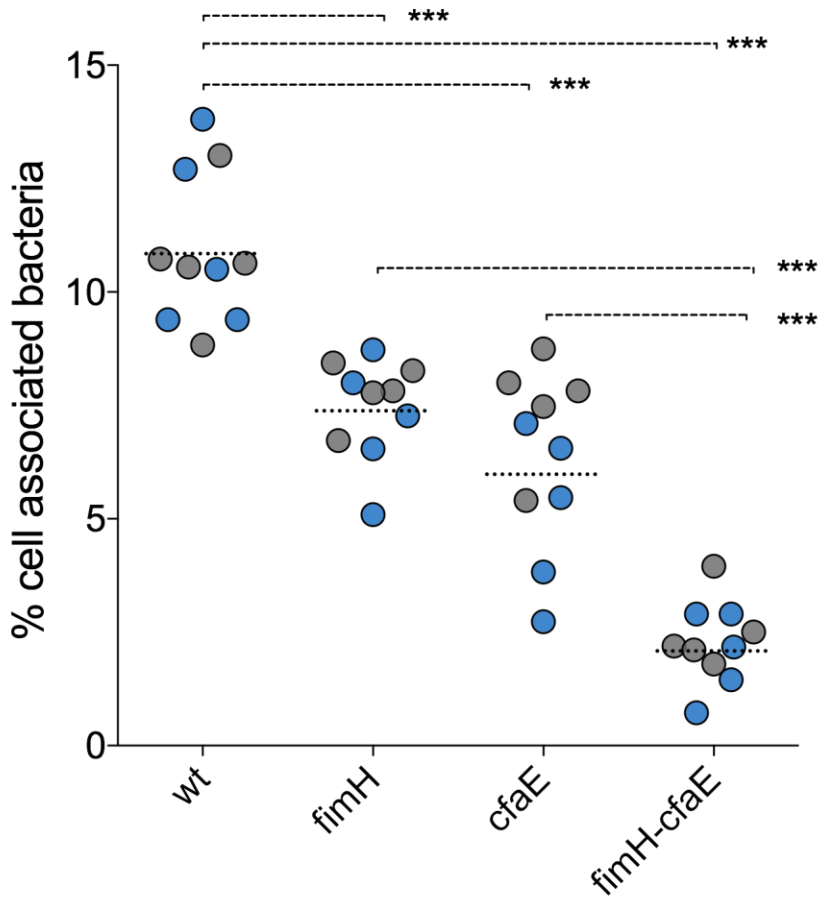
CHAPTER TWO: SUPPLEMENTARY DATA



S1 Fig. Type 1 pili mediated ETEC adhesion to epithelial cells.

a. Adhesion by wild type (wt) bacteria or the *fimH*::Q133K mutant. The percentage of cell associated bacteria represents the proportion of bacteria associated with the epithelial cells 1 h post infection relative to the inoculum. **b.** *Adhesion assays of* wt, *fimA* mutant and *fimA* mutant complemented *in trans* with pFimH, or the vector control plasmid. Yeast agglutination phenotypes for the wt and mutant bacteria are shown below each column of data in the graphs. For data in parts **a** and **b** bars

represent the mean. Error bar, SEM (n = 5). P values were calculated by nonparametric Mann-Whitney test. ** represents $p<0.01$; *$p<0.05$.



S2 Fig. Type 1 pili act in concert with CFA/I fimbriae for optimal adhesion.

In vitro adhesion assay to Caco-2 cells infected with either WT H10407 or different mutants, including *fimH* and *cfaE* single mutants or *fimH-cfaE* double mutants. The percentage of cell associated bacteria represents the proportion of bacteria associated with Caco-2 cells at the end of 1 h relative to the inoculum. Different color dots represent data from different experiments, horizontal dashed lines represent geometric mean values. P values were calculated by nonparametric Mann-Whitney test. *** indicates $p<0.0001$.

S1 Table. Strains used in this study

strain designation	Genotype	Description	Reference
H10407	wild type	ETEC serotype 078:H11, $LT^{+}LST^{+}$	[74]
jf2944	*fimH::kan*	*fimH*; *fimH* gene is replaced with kanamycin resistant gene	This study
jf4622	*fimH::kan* (p*fimH*)	p*fimH*; complemented *fimH* mutants containing wild type *fimH* gene in pFLAG-CTC plasmid, kanamycin and ampicillin resistant	This study
jf4624	*fimH::kan* (p*fimH:Q133K*)	p*Q133K*; complemented *fimH* mutants containing mutant allel of *fimH* gene (*fimH:Q133K*) in pFLAG-CTC plasmid, kanamycin and ampicillin resistant	This study
jf2003	*fimA::kan*	*fimA*; *fimA* gene is replaced with kanamycin resistant	This study
jf2051	*fimA::kan* (p*fimA*)	p*fimA*; complemented *fimA* mutants containing *fimA* gene at the multiple cloning site of ptrc99A plasmid,	This study

		kanamycin and ampicillin resistant	
jf2379	*fimA::kan* (ptrc99A)	*fimA* (control); vector control, complemented *fimA* mutants without the *fimA* gene at the multiple cloning site of ptrc99A plasmid, kanamycin and ampicillin resistant	This study
jf1862	*cfaE::kan*	*cfaE*; *cfaE* gene is replaced with kanamycin resistant gene	[75]
jf2945	*fimH::kan*, *cfaE::cm*	*fimH-cfaE* double mutants; *fimH* gene is replaced with chloramphenicol resistant gene and *cfaE* gene is replaced with kanamycin resistant gene	This study
jf4615	BL21DE3 pLys (p*fimHLD-his*)	BL21DE3 pLys (pETDUET1-fimHLD-his); the lectin domain encoding part of *fimH* gene inserted at multiple cloning site 1 of pETDUET1 expression vector, Ampicillin resistant	This study
jf4617	BL21DE3 pLys (p*fimHLD:Q133*	BL21DE3 pLys (pETDUET1-fimHLD:Q133K-his); mutant allele of	This study

	K-his)	the lectin domain encoding part of *fimH*, *fimH:Q133K* gene inserted in-frame at multiple cloning site 1 of pETDUET1 expression vector, Ampicillin resistant	
jf4638	*fimH*:Q133K	H10407-Q133K; H10407 strain with a point mutation introduced in the mannose binding pocket of *fimH* on the chromosome	This study

S2 Table. Primers used in this study

Primers	Sequence	Description
jf101413.7	CCATTCAGGCAGTGATTAGCATCACCTATACCTACAGCTGAACCCGAAGAGATGATTGTAGTGTAGGCTGGAGCTGCTTC	Forward, *fimH* deletion primer; 60 nucleotides homology tail immediately upstream from *fimH*.p1 region of pKD4
jf101413.8	AAGGGCTAACGTGCAGGTTTTGTAGGTCTGATAGCGTAGCGCCTCAGGTACCAGCATTAGCATATGAATATCCTCCTTA	Reverse, *fimH* deletion primer; 60 nucleotides homology tail immediately downstream from *fimH*.p2 region of pKD4
jf101413.9	TGGCAACACATTGAATACTGG	Forward, *fimH* mutant test primer
jf101413.10	TGCCAGATGCGACGCTGACGC	Reverse, *fimH* mutant test primer
jf062116.1	GAATTTGTAAAGAACCCACGTGTGCAGGATTTGCTGGCAAAGAATGATAAAGGATAAACGGTTTAAACGATATCGGATCCA	Forward, *cfaE* deletion primer; 60 nucleotides homology tail immediately upstream from *cfaE*. nucleotide sequence of *cat* gene
jf062116.2	TTAACAAACAGATTACCTATTTACAATATTGGCGCGCAATAGCGCCAATATTGTTGTTATACTAGTATTACCCTGTTATCC	Reverse, *cfaE* deletion primer; 60 nucleotides homology tail immediately downstream from *cfaE*. nucleotide sequence of *cat* gene
jf042314.1	AGGAGATATACCATGATGAAACGAGTTATTACCCTGT	Forward infusion primer; nucleotides upstream of NcoI

		cloning site of pETDUET1 plasmid.22 nucleotides of *fimH* from the start codon site
jf042314.2	ATGCGGCCGCAAGCT**TTAGTG GTGATGATGGTGATG**GCCGCC AGTGGGCACCAC	Reverse infusion primer; nucleotides downstream of hindIII cloning site of pETDUET1 plasmid. **nucleotides for 6xhistidine tag with stop codon**. last 18 nucleotides of *fimH* without stop codon
jf120814.1	ATATCATATGAAGCTATGAAAC GAGTTATTACCCTGT	Forward infusion primer; nucleotides upstream of HindIII cloning site of pFLAG-CTC plasmid.22 nucleotides of *fimH* from the start codon site
jf120814.2	TGTAGTCGACAGATCTTATTGA TAAACAAAAGTCACG	Reverse, infusion primer; nucleotides downstream of hindIII cloning site of pFLAG-CTC plasmid. last 22 nucleotides of *fimH* with stop codon
jf031814.1	CTCATTAATTGCCGTGCTTATT TTGCGAAAGACCAACAACTATA	Forward, mutagenesis primer for Q133K allele; point mutation is

		underlined
jf031814.2	TATAGTTGTTGGTCTTTCGCAA AATAAGCACGGCAATTAATGAG	Reverse, mutagenesis primer for Q133K allele; point mutation is underlined

S3 Table. Plasmids used in this study

Plasmid	Description	Reference
pFLAG-CTC-*fimH*	Complementation plasmid; *fimH* gene cloned into pFLAG-CTC plasmid, Amp^r	This study
pFLAG-CTC-*fimH:Q133K*	Complementation plasmid; *fimH:Q133K* mutant allele cloned into pFLAG-CTC plasmid, Amp^r	This study
pETDUET1-fimHLD-his	Expression plasmid for purification of FimH lectin domain (FimHLD),Amp^r	This study
pETDUET1-fimHLD:Q133K-his	Expression plasmid for purification of mutated FimH lectin domain (FimHLD:Q133K), Amp^r	This study
pFLAG-CTC	Used as a backbone for complemented plasmid, Amp^r	Sigma
pETDUET-1	Used as a backbone for expression plasmid, Amp^r	Novagen
pKD46	Helper plasmid for λ-Red mediated recombination, Amp^r	[66]
pKD4	Template plasmid for kanamycin resistant gene amplification, Km^r	[66]

Amp^r- ampicillin resistance, Km^r- kanamycin resistance.

CHAPTER THREE

LT induced modification of host glycoproteins alter FimH mediated ETEC-host interactions

ABSTRACT

There is no broadly protective vaccine available against enterotoxigenic *E. coli* (ETEC), the major bacterial cause of acute diarrheal illness in children and travelers. Delivery of ETEC toxins to the target epithelial cells requires direct pathogen-host interaction. Recent studies suggest that ETEC employ multiple factors to directly engage host cells, potentially offering additional targets to interdict toxin delivery. Interestingly, earlier studies reported that heat labile toxin (LT) enhances ETEC-host interactions. Although the mechanism of toxin-induced fluid secretion is fully delineated, how the toxin enhances ETEC-host interactions is undefined. Here, we demonstrate that LT manipulates host pathways in a way that enhances glycoprotein synthesis and shifts glycan synthesis towards production of mannosylated glycans. Additionally, LT increases expression of CEACAM6, a mannosylated glycoprotein receptor of the FimH adhesin, on intestinal epithelial cells, to facilitate ETEC adhesion. Moreover, we found that FimH mediated interactions influence mouse intestinal colonization, particularly in the presence of microbiota. However, the loss of FimH activity increases expression of other bacterial adhesion molecules in mice during transit through the mouse intestinal tract, reinforcing the importance of functionally complementary adhesins. Collectively, data presented here offer a mechanistic explanation of LT-induced enhancement of ETEC engagement with host intestinal epithelia, and identify a novel ETEC-host interaction. These findings expand our understanding of ETEC pathogenesis and may inform a rational approach to ETEC vaccine design.

INTRODUCTION

Enterotoxigenic *E. coli* (ETEC) are among the most frequently isolated bacterial pathogens from cases of moderate to severe diarrheal illness and deaths due to diarrhea in young children in the developing countries (1). ETEC infections are associated with malnutrition (2, 3), poor physical growth (4) and impaired cognitive development (5), and also appear to be more prevalent in malnourished children (2). ETEC are also the major pathogens associated with diarrheal illness among travelers to the countries lacking improved sanitation and safe drinking water (6, 7). Unfortunately, there is no broadly protective vaccine against these pathogens.

Production of heat-labile (LT) and/or heat-stable (ST) enterotoxins are the principal features that define ETEC (8), and pathogenesis requires successful delivery of these toxins to the target epithelial cells (9). LT holotoxin is comprised of pentameric LTB subunit and the active LTA subunit (10). Following delivery, LT binds to GM1 receptors on the cell, is internalized. The LTA subunit constitutively activates adenylate cyclase via ADP-ribosylation of the α-subunit of the host G_s protein (11), which ultimately increases intracellular cyclic-AMP (cAMP) and activates cAMP-dependent protein kinase (PKA). Activated PKA mediated phosphorylation then stimulates several ion transporters, resulting in excessive secretion of fluid and electrolytes into the intestinal lumen (12). Likewise, ST binds to guanylyl cyclase receptors on the cell and increases intracellular cGMP, which stimulates different ion channels (9, 13, 14). In the classical model of ETEC pathogenesis, these bacteria utilize pathovar-specific fimbrial or non-fimbrial adhesins, known as colonization factors or CFs (15, 16) to adhere and colonize

the small intestine for toxin delivery. However, emerging evidence suggests that ETEC engagement of the host is complex and may require collection of adhesins for efficient interaction (17-21).

Bacterial pathogens possess an array of secreted or cell bound adhesive factors which allow them to stick with specific host cells for initial engagement (22-24). Among the most studied bacterial adhesive factors are the heteromeric surface appendages known as pili or fimbriae (24, 25). These pilus structures present the adhesin subunit at the tip to recognize different receptors of the host cell surfaces for adhesion. Many of these receptors are glycoconjugates, including glycolipids and glycoproteins (26, 27), decorated with different sugar residues (glycans), and are ubiquitous on epithelial cell surfaces. Glycoproteins, including mucins, are major constituents of the physical intestinal barrier, comprising of loosely attached thick mucus layer (28, 29), and cell surface bound glycoproteins that form the glycocalyx at the apical surface, protecting the epithelium from invading enteric pathogens (30-32). However, many pathogens, including ETEC, are equipped with multiple mucinases (33, 34) which degrade mucins and may allow their access to the apical glycocalyx.

Glycosylation, a common form of post-translational modification present in all living beings, is essential for the synthesis of secreted and cell surface glycoproteins (35-37). One of the crucial steps in glycosylation is the formation of dolichol-phosphate-mannose (Dol-P-Man), an essential mannosyl donor for multiple glycosyl transferases. Dol-P-Man synthesis is carried out by a mannosyltransferase enzyme, dolichol phosphate mannose (DPM) synthase (38-40). DPM synthase consists of three subunits, DPM1, DPM2 and

DMP3, of which DPM1 is the active subunit (41) that catalyzes the reaction between dolichol-phosphate and GDP-mannose to generate Dol-P-Man. Dol-P-Man is essential for glycosyl phosphatidylinositol (GPI) membrane anchor synthesis, O- and N-glycosylation, as well as mannosylation (38).

ETEC adhesins characterized thus far are lectins that interact with different glycans presented on cell surface glycoconjugates (42-44). Therefore, host surface glycoproteins likely play vital roles in ETEC adhesion, and changes in host glycosylation processes could alter surface glycan presentation on epithelial cells to modulate ETEC-host interactions. Cyclic-AMP influences glycoprotein secretion (45-48). Because LT increases host intracellular cAMP (9) and enhance ETEC adhesion to intestinal epithelium (49, 50), ETEC infection could modulate expression of host cell surface glycoproteins, favoring enhanced interactions with host. Early studies reported that cAMP-dependent protein kinase A-mediated phosphorylation activates DPM1 mannosyltransferase and thus increases Dol-P-Man formation (48, 51, 52), potentially supporting synthesis of mannosylated glycoproteins (40, 53). In addition, it has been reported that cAMP increases expression of GPI-anchored carcinoembryonic antigen-related cell adhesion molecule-6 (CEACAM6), a receptor for the FimH adhesin of type 1 pili (54). As aype 1 pili of multiple *E. coli* interact with CEACAM6 (55, 56) and these pili facilitate ETEC interaction with intestinal epithelial cells (57), ETEC may also employ CEACAM6 for adhesion to host.

Microbiota contribute significantly in protecting the intestinal epithelium from enteric infections (58). For successful colonization, ETEC must overcome the ‘colonization

resistance' offered by resident microbiota (59). Additionally microbiota has profound effects on gut epithelial maturation and nutrition uptake (60, 61) and also actively participates in degradation of glycans present in the intestinal mucosa (62, 63), perhaps contributing in shaping the intestinal surface glycan structure. Therefore, since toxin-induced intracellular cAMP stimulates mucosal glycoprotein synthesis and secretion (43, 45, 46), suggesting probable alteration of cell surface glycans, LT-mediated modification of host cell surface glycans could also alter the intestinal microbiota structure, and indirectly influence ETEC-host interactions. In this study we present data which support the hypothesis that LT alters cell surface glycoprotein presentation and thus influences FimH mediated ETEC-host interactions.

RESULTS

LT toxin alter expression of genes involved in glycosylation pathway

Because LT treatment has been shown to enhance intestinal epithelial mucin production (43), and because mucins are heavily glycosylated, we questioned questioned whether the glycan profile of surface glycoproteins might also be altered following toxin exposure. To address this question we first examined the expression profile of the different genes involved in glycosylation pathways. RNAseq analyses were performed on Caco-2 intestinal cells treated with LT or mutant LT (mLT, inactivated by introduction of a point mutation, R192G) (64) and compared these to untreated cells. Cluster analysis based on Pearson distance did not show any difference in gene expression profile of cells treated with mLT and untreated cells, but cells treated with LT demonstrated differential expression of many genes, including genes involved in

glycosylation processes (Figure S1a-b). Intriguingly, expression of *DPM1*, which encodes the active enzymatic subunit of DPM synthase, was increased in LT-treated cells compared to either mLT-treated cells or untreated cells (Figure 1a, b). *DPM1* expression in cells treated with mLT or in untreated cells was comparable, suggesting that the enzymatic activity of LT is critical for increased expression of *DPM1*. We validated these RNAseq data by quantitative RT-PCR analyses (Figure 1b, inset). Consistent with the known effect of cAMP-dependent protein kinase (PKA) mediated phosphorylation and activation of DPM1 (51), we observed toxin dependent increase in *DPM1* gene expression, supporting activation of glycoprotein synthesis pathways following LT treatment.

Among other genes of dolichol-linked oligosaccharide precursor synthesis process, *DOLK*, *DOLPP*, *ALG1*, *ALG2*, *ALG7* and *ALG11* were also significantly increased in LT treated cells (Figure 1c). *DOLK* encodes dolichol kinase which phosphorylates ER membrane-anchored dolichol to generate dolichol-phosphate (Dol-p), followed by addition of two N-acetyl glucosamine residues facilitated by dolichol phosphate-N-acetylglucosamine transferase encoded by *ALG7*, and N-acetylglucosamine transferases encoded by *ALG13*/*ALG14*. Subsequently, 5 mannose residues are added by mannosyl transferases, encoded by *ALG1*, *ALG2* and *ALG11* genes, yielding the initial glycan structure, Dol-pp-(GlcNAc)2-Man5, for further processing.

Mannosidases are also involved in intermediary steps of the N-glycosylation pathway and changes in the activity of these enzymes might alter glycan composition (65, 66). Disruption of glycan processing by alpha-mannosidase alters glycosylation patterns (67,

68)**.** Earlier we demonstrated that inhibition of α-mannosidase class 1 (MAN1A1) enzyme with kifunensine significantly increased surface expression of mannosylated glycoproteins on epithelial cells, allowing enhanced binding of mannose specific FimH adhesin and promoting ETEC adhesion (57). Interestingly, in RNAseq analyses, we observed decreased expression of two key mannosidase encoding genes, *MAN1A1* and *MAN2A1*, in cells treated with LT but not in cells treated with mLT (Figure 2a). The MAN1A1 mannosidase trims the alpha-1,2-linked mannose residues from $Man_9GlcNAc_2$ to produce $Man_5GlcNAc_2$ for further processing to hybrid and complex glycans (69, 70). The Man2A1 mannosidase catalyzes the first committed step in the biosynthesis of complex N-glycans, cleaving the alpha-1,3-linked and alpha-1,6-linked mannose residues of $Man_5GlcNAc_2$ to generate $Man_3GlcNAc_2$ core structure for complex glycan synthesis (71, 72). Therefore, decreased expression of these two mannosidase genes could significantly alter cell surface glycan architecture.

LT favors presentation of highly mannosylated surface glycans

Because LT modulates expression of multiple enzymes involved in glycosylation pathways, these changes will likely impact glycan structure of surface glycoproteins. Therefore, we examined the surface glycan pattern of intestinal cells treated with LT or mLT and compared with untreated cells. N-glycans expressed on intestinal cell surfaces were recovered and analyzed by nano-LC/MS, revealing a shift toward more heavily mannosylated glycans in cells treated with LT than in cells treated with mLT or untreated cells (Figure 2b). Similarly, ETEC infection induced surface expression of high mannose glycoproteins, as we observed significant increases in high mannose glycan

content in cells infected with ETEC than in uninfected cells (Figure 2c). In line with these observations, binding of Galanthus nivalis agglutinin (GNA), a mannose lectin (73), was enhanced when cells were treated with LT compared to untreated cells. Therefore, these data strongly suggest LT increases expression of mannosylated glycoproteins on intestinal epithelial cells.

LT alters expression of CEACAMs on intestinal epithelial cells

Glycoproteins of the CEACAM family, including CEACAM1, CEACAM5, CEACAM6 and CEACAM7, are integral part of the intestinal glycocalyx (74, 75). Of these, CEACAM5 and CEACAM6 are mannosylated glycoproteins (55). Intriguingly, RNAseq analyses of Caco-2 cells treated with LT demonstrated altered expression of multiple genes of the CEACAM family (Figure 3a). While expression of *CEACAM18* and *CEACAM19* genes were decreased, expression of *CEACAM1*, *CEACAM5*, *CEACAM6* and *CEACAM7* genes were significantly increased in cells treated with LT compared to cells treated with mLT or in untreated cells. To validate *CEACAM5* and *CEACAM6* upregulation, we examined the expression of these genes by quantitative RT-PCR (Figure 3b). The expression of both CEACAMs was indeed increased in LT-treated cells compared to untreated cells or those treated with mLT, suggesting that the enzymatic activity of the toxin is necessary for induction of these CEACAMs. In agreement with these transcription data, increased amounts of CEACAM5 and CEACAM6 were detected using monoclonal antibodies specific to CEACAM5 (not shown) and CEACAM6 (Figure 3c, top panel). Similarly, we observed increased CEACAM6 production following

forskolin induction of cAMP, supporting a role for this second messenger in modulating glycoprotein expression (Figure 3c, bottom panel).

ETEC interacts with intestinal CEACAM6

Because earlier studies demonstrated that *E. coli* binds to mannosylated CEACAM6, we hypothesized that LT-induced enhancement of CEACAM6 expression in intestinal epithelial cells might facilitate ETEC adhesion. Intriguingly, cells infected with ETEC demonstrated localized increases in expression of CEACAM6 around adherent ETEC (Figure 4a). Since FimH interacts with CEACAM6 and ETEC utilize FimH mediated interaction for adhesion, increased expression of CEACAM6 around ETEC could facilitate adhesion. To examine the contribution of CEACAM6 in ETEC adhesion, we used CRISPR-Cas9 to generate Caco-2 cells lacking CEACAM6 (*CEACAM6*$^{-/-}$). After validating *CEACAM6* deletion (Figure 4b), we assayed GNA lectin binding to the *CEACAM6*$^{-/-}$ cells (Figure 4c). As expected, significant reduction of GNA binding was observed to *CEACAM6*$^{-/-}$ cells compared to parent Caco-2 cells (*CEACAM6*$^{+/+}$). Interestingly, LT treatment of *CEACAM6*$^{-/-}$ cells increased GNA binding, suggesting that LT induces other mannosylated glycoproteins on *CEACAM6*$^{-/-}$ cells. ETEC adhesion to *CEACAM6*$^{-/-}$ cells was significantly reduced compared to parent Caco-2 cells, while *fimH* mutants to *CEACAM6*$^{-/-}$ and *CEACAM6*$^{+/+}$ cells was comparable (Figure 4d), implying that ETEC interaction with CEACAM6 is probably FimH mediated. Additionally, in line with GNA binding data, adhesion of WT ETEC to *CEACAM6*$^{-/-}$ cells was significantly higher than *fimH* mutant, suggesting that FimH of ETEC may also interact with other mannosylated proteins present on *CEACAM6*$^{-/-}$ cells.

FimH mediated interactions influence ETEC colonization of mouse intestine

In order to investigate the contribution of FimH in intestinal colonization by ETEC, we first examined the colonization dynamics of *fimH* mutants in mice. CD1 mice were infected with either *fimH* mutants or wild type ETEC (WT), and at 24 hrs post infection intestinal colonization in different niches including jejunum, ileum, colon and feces was examined. In mice infected individually with WT or the *fimH* mutant, intestinal colonization was comparable (Figure 5a). Likewise, comparable levels of ETEC shedding were observed in feces of WT or mutant infected mice (Figure 5a). Additional groups of mice were infected with either the WT or *fimH* mutant and observed over 10 days post infection for fecal shedding. No significant difference was observed in overall fecal shedding between *fimH* mutant and WT over the follow-up period (Figure 5b). Fecal shedding of both WT and *fimH* mutants fell steadily over a week, demonstrating typical self-limiting colonization of ETEC (76). Interestingly however, in competition assays where mice were co-infected with *fimH* mutant and WT, we observed increased colonization and shedding of the *fimH* mutant relative to the WT (Figure. 5c).

Intestinal microbiota influence FimH-mediated colonization

The intestinal lumen is heavily colonized by microbiota (77), which inhibit the colonization of invading intestinal pathogens (58, 78). Some members of the microbiota degrade glycans found in host mucus secretions or shed epithelial cells (79) that could change the availability of intestinal glycans and impact pathogen-host interactions. Therefore, the streptomycin treatment in our standard infection protocol, which reduces intestinal microbiota, could affect pathogen-host interactions. To test this, intestinal

colonization by *fimH* mutant was examined in mice treated with or without streptomycin. As predicted, overall colonization of both WT ETEC and *fimH* mutant were decreased in untreated mice, as we recovered significantly fewer bacteria in the feces from untreated mice than from streptomycin treated mice (Figure 6a). These findings are in agreement with the inhibitory role of microbiota in ETEC colonization. Intriguingly, in competitive colonization assays, we observed that intestinal colonization of mice (both streptomycin treated and untreated) by *fimH* mutant was more than WT ETEC (Figure 6b).

We further tested the impact of intestinal microbiota on FimH mediated colonization by ETEC. For this, mice were co-infected with ampicillin resistant WT and *fimH* mutant in competitive colonization assays. To control intestinal microbiota, ampicillin was supplied in the drinking water as indicated. High colonization titers of both WT and *fimH* mutant were maintained over the initial ampicillin treatment period (days 1-14) (Figure 6c). However, removal of ampicillin treatment rapidly decreased ETEC colonization (days 14-21), suggesting recovery of microbiota which perhaps inhibited ETEC colonization. Re-administration of ampicillin, however, reinstituted ETEC colonization (days 21 to 35). Intriguingly, during this period we observed significantly less shedding of the *fimH* mutant than the WT ETEC, and a lower competitive index of *fimH* mutant over WT ETEC (Figure 6d), implying possible contribution of FimH in ETEC colonization in the presence of microbiota.

Increased expression of multiple adhesion related genes in *fimH* mutants recovered from mice

To begin to understand the determinants of increased colonization of *fimH* mutants in co-infections, we examined the expression profile of genes that were known to be involved in ETEC colonization. For this, we performed RT-PCR analyses on fecal samples collected from mice infected with WT or *fimH* mutants. Intriguingly, we detected increased expression of multiple genes, including *fimA*, and *cfaB* that encode the major structural subunit of type 1 pili and CFA/I fimbriae, respectively (Figure 7a). Additionally, we examined fimbrial production of the *fimH* mutants. Using electron microscopy we observed that while the majority of the *fimH* mutant bacteria were non-fimbriated (Figure 7b), some cells in this population were extensively fimbriated (Figure c). Examination of pili prepared from both bacterial cultures showed a major pilin subunit (Figure 7d). However, only pili prepared from WT were reactive to anti-type 1 pili antibody on immunoblots (Figure 7d), indicating that *fimH* mutant are making other pili. Mass spectrometry analysis of the fimbrial preparation from the *fimH* mutant identified CfaB, the major structural subunit of CFA/I fimbriae, suggesting that *fimH* mutants are producing CFA/I fimbriae. In addition to these fimbrial genes, we detected increased expression of *pqiB* which encodes a homologue of the multivalent adhesion molecule 7 (Mam7) (Figure 7a). Mam7 is important for adhesion (80, 81) and pathogenesis of many Gram-negative pathogens including *Vibrio cholerae* and enteropathogenic *E. coli* (81). Therefore, these data support the idea that relative to WT ETEC *fimH* mutants overexpress multiple adhesion related genes.

Altered expression of adhesion related genes in fimH mutants promotes enhanced colonization of mice

Increased expression of multiple adhesion related genes could transform *fimH* mutant into a better colonizer than WT ETEC in mice. To investigate whether enhanced expression of different adhesion related factor(s) were involved in increased colonization of *fimH* mutants, we generated multiple mutants by sequential deletion of genes that were overexpressed in *fimH* mutants. Analogous to FimH of type 1 pili, CfaE is the tip adhesin of CFA/I fimbriae required for interactions (82) and *pqiB* encodes a Mam7 adhesin. Therefore, we tested *fimH-cfaE* and *fimH-pqiB* double mutants, as well as *fimH-cfaE-pqiB* triple mutants, in competitive colonization experiments with WT. Surprisingly, like *fimH* mutants, both *fimH-cfaE* and *fimH-pqiB* double mutants also outcompeted WT ETEC in competitive colonization of mice (Figure 8). Interestingly, however, *fimH-cfaE-pqiB* triple mutants colonized less efficiently than WT ETEC (Figure 8). Therefore, increased expression of CFA/I fimbriae and homologue of Mam7 adhesin in *fimH* mutants could account for increased colonization of mice by the *fimH* mutant. Overall, these experiments demonstrate the enormous complexity of ETEC-host interactions during pathogenesis.

DISCUSSION

Enterotoxigenic *E. coli* adhere to and colonize intestinal epithelia where these bacteria deliver LT and/or ST to cognate receptors (9). Delivery of these toxins to target epithelial cells requires direct host contact (83). Like other pathogenic *E. coli*, ETEC are equipped with numerous variety of virulence factors that include both highly conserved (21, 34) as well as pathotype-specific features (33). Included among these virulence factors are adhesins (20, 43, 57, 84) that enable ETEC to make the necessary contact

with host for toxin delivery. Since adhesins are mostly lectins that recognize glycan structures of glycoconjugates, modification of host cell surface glycoproteins could influence ETEC-host interactions. Collectively, data presented here suggest that LT modifies host glycosylation processes and enhances expression of highly mannosylated cell surface glycoproteins that could influence ETEC interactions with intestinal epithelium. These findings support recent observations of ETEC-induced changes in target cells to enhance interactions with the host (43, 85).

Because cAMP is a second messenger in myriad cell signaling pathways (86) and activates PKA that phosphorylates any protein with an exposed R-X-X-pS/T motif (87), LT-induced cAMP could possibly, directly and/or indirectly, stimulate multiple host responses (86, 88, 89), including glycosylation processes. Indeed, data obtained from RNASeq and mass spectrometric analyses demonstrated that, in addition to targeting host glycosylation processes, LT also enhances expression of multiple cell surface glycoproteins, including carcinoembryonic antigen-related cell adhesion molecules (CEACAMs).

CEACAMs, including CEACAM1, CEACAM5 (also known as CEA or CD66e), CEACAM6 (also known as NCA or CD66c) and CEACAM7 are highly glycosylated constituents of the intestinal apical glycocalyx (74, 75) and influence pathogen-host interactions (56, 90-93). Interestingly, the FimH adhesin of type 1 pili of *E. coli* binds to CEACAM6 (54, 55, 92). Our observations of increased CEACAM6 expression following LT treatment and FimH-mediated ETEC association with CEACAM6 (Figure 4) inform a novel interaction of ETEC infection that might influence the outcome of these

interactions. Since intestinal CEACAM6 is distributed in multiple locations- membrane bound on epithelial cells or on microvesicles, in filaments making up the glycocalyx of intestinal epithelium, and as secretory components of goblet cells secreted together with mucins (94), ETEC interactions with these different forms of CEACAM6 could therefore impact ETEC adhesion. For example, while attachment to cell bound CEACAM6 may confer adhesion, attachment of ETEC to unbound CEACAM6 could facilitate clearance from the intestine. Since cAMP enhances goblet cell secretion (89), LT could increase secretion of luminal CEACAM6 that might serve as decoy receptor. Consequently, enhancement of CEACAM6 production following LT treatment could be a host defense mechanism. Alternatively, ETEC could use these interactions to facilitate adhesion with host at the early stages of infection, as the infection progress, LT-induced induction might increase secreted CEACAM6 that ETEC could exploit to detach from the host and exit in the stool to start the next round of infection. Therefore, although FimH-CEACAM6 mediated interactions facilitate colonization of human ileum by AIEC (54, 95), the contribution of this interaction for ileal colonization by ETEC is yet to be fully understood.

In theory, alteration of the intestinal glycan landscape following ETEC infection could perturb composition of the microbiota and hinder intestinal homeostasis, including nutrient absorption. Indeed, ETEC infections are associated with malnutrition in children (3), and recently, one study reported that ETEC as well as *Vibrio cholerae* infections alter intestinal microbial community structure (96). Since microbiota degrade mucosal

glycoproteins (97), presence or absence of microbiota could change the architecture and availability of intestinal glycans, and thereby impact ETEC-host interactions.

In order to cause infection, enteric pathogens must breach host innate defense mechanisms and overcome microbiota-exerted inhibition of colonization, which ETEC accomplish by employing multiple virulence factors (18-20, 33, 34, 43). Deletion of any of these factors compromises the dynamics of ETEC-host interactions. While the increased intestinal colonization of mice by *fimH* mutant observed in the present study is counterintuitive, our studies suggest that ETEC are equipped with mechanism(s) to overcome the loss of FimH activity. Importantly, we detected overexpression of multiple adhesion factors, and identified induction of a novel Mam7 adhesin of ETEC in *fimH* mutants recovered from feces, supporting the hypothesis that ETEC possess functionally complemented factors and/or regulatory mechanisms to optimize infection. Together, these data illustrate the complexity of ETEC colonization which involves ETEC, host and the resident microbiota. On the other hand, the relevancy of intestinal colonization data obtained from mice to the human host is limited by several factors: first, mice do not make CEACAM6, second, intestinal mucins produced by mice and humans are somewhat different, and third, intestinal microbiota as well as diet are also different, and may affect intestinal glycan content. For all these reasons, the mouse model may not capture all the ETEC-host interactions presented during human intestinal colonization.

In summary, we identified several novel ETEC host interactions, including modification of host glycosylation pathways, and detected interaction between a highly conserved

FimH adhesin and host CEACAM6 in ETEC pathogenesis, suggesting a mechanism for LT-mediated enhancement of ETEC adhesion. Additionally, found that Mam7 adhesin might participate in ETEC-host interactions. Data presented here also suggest intricate interactions between ETEC and intestinal microbiota during colonization. Overall, the findings described here underscore the highly complex nature of ETEC-host interactions and expand our understanding of ETEC pathogenesis in ways that foster rational vaccine design approaches that target these globally important pathogens.

METHODS

Cell culture and treatment

Caco-2 intestinal cell (ATCC) culture was maintained in MEM media supplemented with 20% FBS. In order to investigate the effect of LT toxin on intestinal epithelial cells, Caco-2 cells were seeded at ~0.5 x10^5 cells/well of 96-well cell culture plate in MEM media supplemented with 20% FBS (Gibco). Cells were grown at 37˚C in incubator with 5% CO_2 for 4 days with media changed every 2 days. Cells were treated with different toxins, including, holotoxin (LT), mutant toxin (mLT) and B subunit of LT (LTB), by replacing the culture medium with fresh medium supplemented with 0.1 µg/ml final concentration of toxin. Following overnight treatment, cells were washed with pre-warmed PBS and processed for subsequent analysis.

RNA isolation, cDNA synthesis and RT-PCR

Caco-2 cells treated with toxins were processed for RNA extraction using illustra RNAspin mini (GE Healthcare) RNA extraction kit. cDNA synthesis was done using SuperScript VILO cDNA synthesis kit (Invitrogen) by using 0.1 µg of total RNA. Equal

amount of cDNA was used to perform RT-PCR for comparative expression analysis. All reactions were performed in triplicate, including GAPDH control, using SYBR Green based master mix (Thermo Fischer) and samples were run on Applied Biosystems® 7500 Real-Time PCR System (Applied Biosystem) and analyzed with ViiA7 software (v1.2.4). Normalized expressions were calculated by ΔC_T method.

Mutagenesis and knock out

Double mutant and triple mutant (table 1) were constructed using lambda red mediated recombination as previously described (98). To construct *fimH-cfaE* and *fimH-pqiB* double mutant gene *cfaE* and pqiB, respectively, were deleted and for *fimH-cfaE-pqiB* triple mutant gene *cfaE* and *pqiB* were deleted sequentially from the *fimH* mutant. For deletion of *cfaE* gene primers jf101413.7 and jf101413.8 and for deletion of *pqiB* gene primers jf060716.1 and jf060716.2 (table 2) were used to amplify the kanamycin resistance cassette from pKD4 plasmid with 60-bp tails corresponding to the DNA sequence immediately upstream and downstream of respected genes. The resulting amplicon was then introduced into *fimH* carrying the pKD46 helper plasmid for lambda red-mediated homologous recombination and mutants were selected on 50 µg/ml Kanamycin containing LB-agar plate and screened for mutant by PCR. In order to delete *CEACAM6* we used CRISPR/Cas gene modification system, generating *CEACAM6*$^{-/-}$ cells.

Type 1 pili extraction and detection

Bacteria culture, grown in type 1 pili inducing conditions, was processed for pili extraction by following a previously described method (57). Pili extracts were separated

on SDS-PAGE minigel (Bio-Rad) and either stained with SYPRO Ruby protein gel stain (Invitrogen) or transferred to nitrocellulose membranes (Bio-Rad) using a Mini Trans-Blot electrophoretic cell (Bio-Rad) for 60 min at 100 V. Following blocking with 5% milk-PBS supplemented with 0.05% Tween 20 (Pierce), the membrane was incubated with anti-type 1 pili primary raised in rabbit (1:5,000) and HRP conjugated anti-rabbit secondary (1:5,000) antibodies. Chemiluminescent substrate (Clarity Western ECL substrate, Bio-Rad) was used for detection.

Adhesion assay

Bacteria were grown at type 1 pili inducing conditions as described previously (57). For adhesion assay, cultures were diluted at 1:10 in pre-warmed cell culture media before infecting cells. Inoculums were serially diluted and plated on Luria agar for CFU count. Following inoculation cells were incubated at 37°C and 5% CO_2 for 1 h, washed with pre-warmed tissue culture medium 3 times with gentle shaking (100 rpm) for 1 min each. Infected cells were then lysed in 0.1% Triton X-100 for 5 min, and the cell associated bacteria were recovered by plating lysates onto Luria agar for CFU count and expressed as percent cell associated bacteria.

Fluorescence microscopy

In order to investigate ETEC association with CEACAM6 polarized epithelial cell cultures, grown on trans-well filter, were infected with WT or *fimH* mutant ETEC. Following infection, cells were washed 3 times with pre-warmed tissue culture medium with gentle shaking (100 rpm) for 1 min each to remove unbound bacteria (57). Cells were then fixed with 4% paraformaldehyde for 30 min at room temperature (RT),

washed with 3 times with PBS and blocked with 1% BSA-PBS for 30 min at RT. Anti-CEACAM6 monoclonal antibody raised in mouse (Santa Cruz) and anti-O78 antibody raised in rabbit were used for detection of CEACAM6 and bacteria, respectively. Fluorescent conjugated anti-mouse and anti-rabbit secondary antibodies were used to visualize CEACAM6 and cell associated bacteria by fluorescent microscopy.

Transmission electron microscopy

For negative staining, bacteria were allowed to absorb onto glow discharged formvar/carbon-coated copper grids for 2 min. Grids were then washed with PBS, fixed with 1% glutaraldehyde, and stained with 1% aqueous uranyl acetate (Ted Pella Inc., Redding CA) for 1 min. Excess liquid was gently wicked off and grids were allowed to air dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA) equipped with an AMT 8 megapixel digital camera (Advanced Microscopy Techniques, Woburn, MA).

Mouse intestinal colonization

To investigate the contribution of FimH mediated ETEC-host interactions in colonization, mice were challenged with ETEC as previously described (50). Briefly, 5-8 week old female mice (CD1) were pretreated with streptomycin (5 g/liter) in drinking water for 24 h to suppress microbiota mediated colonization resistance (99), unless otherwise stated. For single infection mice were inoculated with either WT or *fimH* mutant by oral gavage. Another group of mice were co-infected with WT and *fimH* mutant mixed at 1:1 ratio. To examine the influence of streptomycin treatment on FimH mediated interactions, a group of untreated mice were also co-infected. Mice were sacrificed at

specified times, different intestinal sections were collected and lysed in 5% saponin, and lysates were plated for CFU count. Since fecal shedding reflects intestinal colonization, for colonization kinetics, fecal samples were collected every day for 10 days from a group of single infected mice. To investigate the contribution of microbiota on FimH mediated interactions, mice infected with WT and *fimH* mutant (both strains harboring plasmid with ampicillin resistant gene) were continuously treated with ampicillin (100 mg/liter) in drinking water for 2 weeks, followed by 1 week of without ampicillin treatment to allow microbiota recovery, followed by additional 2 weeks of ampicillin treatment. Fecal samples were collected at different days as indicated, for CFU count.

Bacterial gene expression analysis

Fresh fecal samples from mice infected with either *fimH* or WT ETEC were collected in PBS on ice, resuspended and centrifuged at 350 × g to remove debris. A 500 µl of suspension was mixed with equal amount of Trizol (Invitrogen) and stored immediately at -80˚C. RNA was isolated per manufacturer's protocol and cleaned up using RNeasy kit (Qiagen), followed by DNAse I digestion. Conventional PCR for *arcA* (a housekeeping gene) was used to confirm the removal of DNA (data not shown). cDNA synthesis and RT-PCR were done as above.

CHAPTER THREE: TABLES

Table 1: List of strains used

Strain designation	Genotype	Description	Reference
H10407	wild type	ETEC serotype 078:H11, $LT^{+}LST^{+}$	(100)
jf2944	*fimH::kan*	*fimH*; *fimH* gene is replaced with kanamycin resistant gene	(57)
jf2945	*fimH::kan*, *cfaE::cm*	*fimH-cfaE* double mutants; *fimH* gene is replaced with kanamycin resistant gene and *cfaE* gene is replaced with chloramphenicol resistant gene	(57)
jf4674	*fimH::kan, pqiB::cm*	*fimH-pqiB* double mutants; *fimH* gene is replaced with kanamycin resistant gene and *cfaE* gene is replaced with chloramphenicol resistant gene	This study
jf2944	∆*fimH, cfaE::kan, pqiB::cm*	*fimH-cfaE-pqiB* triple mutants; *fimH* gene is deleted and *cfaE* gene is replaced with kanamycin resistant gene and *pqiB* gene is replaced with chloramphenicol resistant gene	This study

Table 2: List of primers

Mutagenesis primers		
Primers	**Sequence**	**Description**
jf062116.1	GAATTTGTAAAGAACCCACGT GTGCAGGATTTGCTGGCAAAG AATGATAAAGGATAAACGGTT TAAACGATATCGGATCCA	Forward, *cfaE* deletion primer; 60 nucleotides homology tail immediately upstream from *cfaE*. nucleotide sequence of *cat* gene
jf062116.2	TTAACAAACAGATTACCTATTT ACAATATTGGCGCGCAATAGC GCCAATATTGTTGTTATACTAG TATTACCCTGTTATCC	Reverse, *cfaE* deletion primer; 60 nucleotides homology tail immediately downstream from *cfaE*. nucleotide sequence of *cat* gene
jf060716.1	ATGAGTCAGGAAACGCCCGCT TCGACGACTGAAGCGCAGATT AAAAATAAACGCCGTATCGTT TAAACGATATCGGATCCA	Forward, pqiB deletion primer, 60 nucleotides homology tail immediately upstream from *pqiB*. nucleotide sequence of *cat* gene
jf060716.2	TTATTTGGGAAGCGCAGTACC CCATTCACGCCACTCTTTCGG TTCACTTTCCTGCAACAGACT AGTATTACCCTGTTATCC	Reverse, *pqiB* deletion primer; 60 nucleotides homology tail immediately downstream from *pqiB*. nucleotide sequence of *cat* gene
RT-PCR primers		
Gene	**Primer sequences**	
Bacteria	**Forward**	**Reverse**

etpA	TACCGTGTTCATCAGCATACC	GGTGACAGGAGATGTGGTTAAT
fimH	GTGATGTTTCTGCTCGTGATG	GTTGTGCCGGAGAGGTAATA
cfaB	GAGTGCTTCAGCAGTAGAGAAA	TGATGCGGGAGAATAAGCTAAC
eatA	CACCAATCTGAACGTGGGTATAG	TTACGGGATAGTCAGGGAGATG
fimA	GCTGGATGGTGCGTCATTTA	GCATTAGCAGCACCTGGGGTTG
eaeH	GAAGGATGCGTACGGGAAC	CCGCTAAACACTGGTGCAT
matA	CATCACGGTATCGCCAGTTT	GGTCTATTTGACGTGGCTATCG
ybgD	ATCGCTGTGATGTGACTGATTA	GCTATTCACTGCACCTGTTTATG
yadN	CGTTGGCGCTAAAGCTAAAC	AGCGATATTAACGCCGTCAG
yehD	AAGGCGATAGCCAGTAGCAA	CCACACGCATAACCAGAGAA
yraH	GATAATGGCACCGTCCCTAA	CGGGATCTGATAAACCGTGT
yfcV	GTTCCGGACGATCACAAACT	GCCGCTATTTGCAGAAGAAA
pqiB	TCGTAAACTCACCAGCAAAGG	AGAAACTCAACGCCATCCAG
arcA	CGAAATCGGTGCAGATGAC	TGCACGAATCGTCAGTTCA
Human		
CEACAM6	CACCGTCGGCATCACGA	GAAGAATTCAGGGTCTGGTCCA
DPM1	ATGGATGCTGATCTCTCACACC	CCATTTCCTTTGTAGCGAGTTC
GAPDH	TGACAACGAATTTGGCTACAGC	TGATGGTACATGACAAGGTGC

CHAPTER THREE: FIGURES

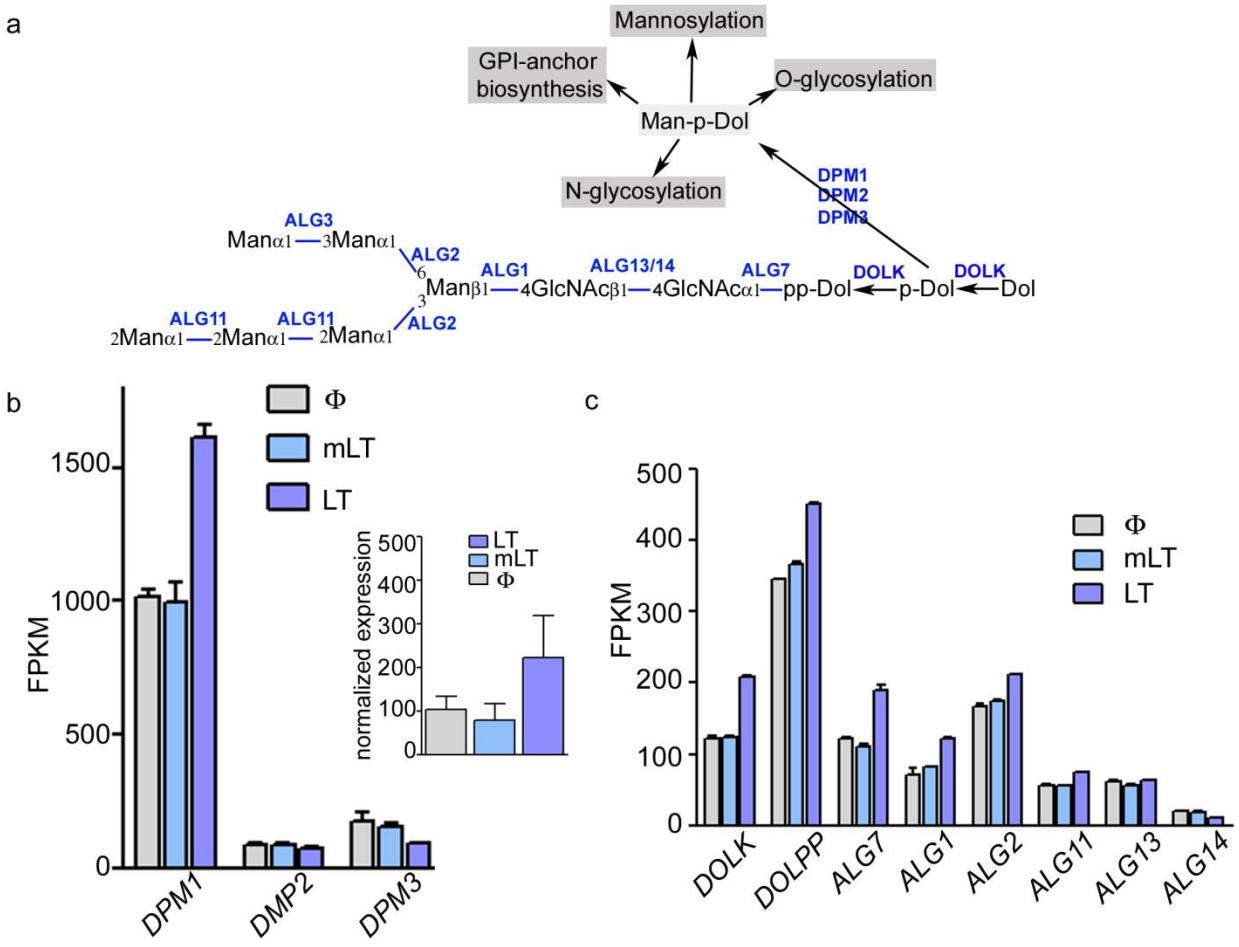


Figure 1. LT modulate transcription of genes involved in glycan synthesis pathway.

a) Schematic diagram of glycan precursor synthesis pathway. Enzymes involved in the different steps of the pathway are shown in blue. b) RNAseq data showing expression of different subunit of DPM synthase in cells treated with holotoxin (LT), mutant toxin (mLT) or untreated (Φ). Each bar represents mean FPKM (Fragments Per Kilobase of

transcript per Million mapped reads) value of duplicate samples. Inset showing RT-PCR analysis of active subunit, *DPM1*, of DPM syntahse. Bar represents % gene expression normalized to *GAPDH* (n=3). c) RNAseq analysis of genes involved in glycan precursor formation. Bar represent mean±SD of duplicate samples.

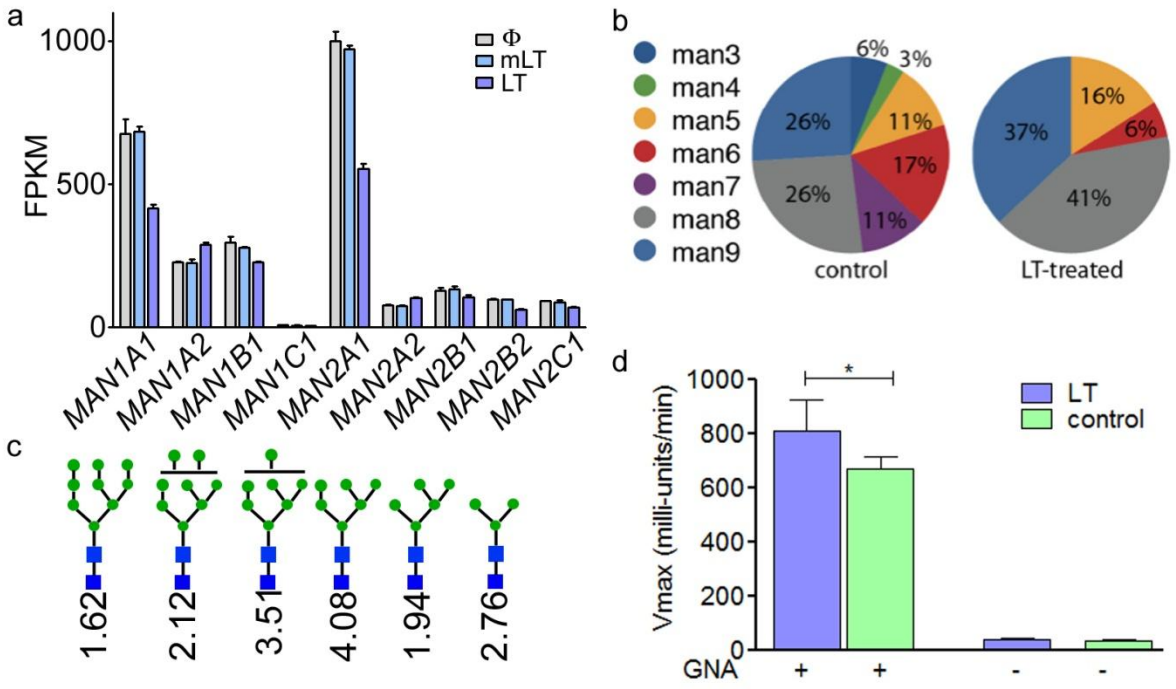


Figure 2. LT modify host glycan synthesis towards high mannose glycan.

a) Transcription analysis of different mannosidase enzymes involved in glycosylation process. Data showing FPKM value generated from RNAseq analysis of cells treated with holotoxin (LT), mutant toxin (mLT) or untreated (Φ). Bar represent mean±SD of duplicate samples. b) Mass spectrometric analysis of surface expressed mannosylated glycans recovered from intestinal cells treated with LT or from untreated cells (control). Pie chart presenting changes in abundance of different mannosylated glycans following LT treatment. c) Types of mannosylated glycans recovered following ETEC infection. Number indicates fold change in abundance relative to uninfected cells. d) Data showing mannose specific GNA lectin binding to cells treated with LT. Bar represents mean±SD (n=5). P value was calculated by nonparametric Mann-Whitney test. *p<0.05.

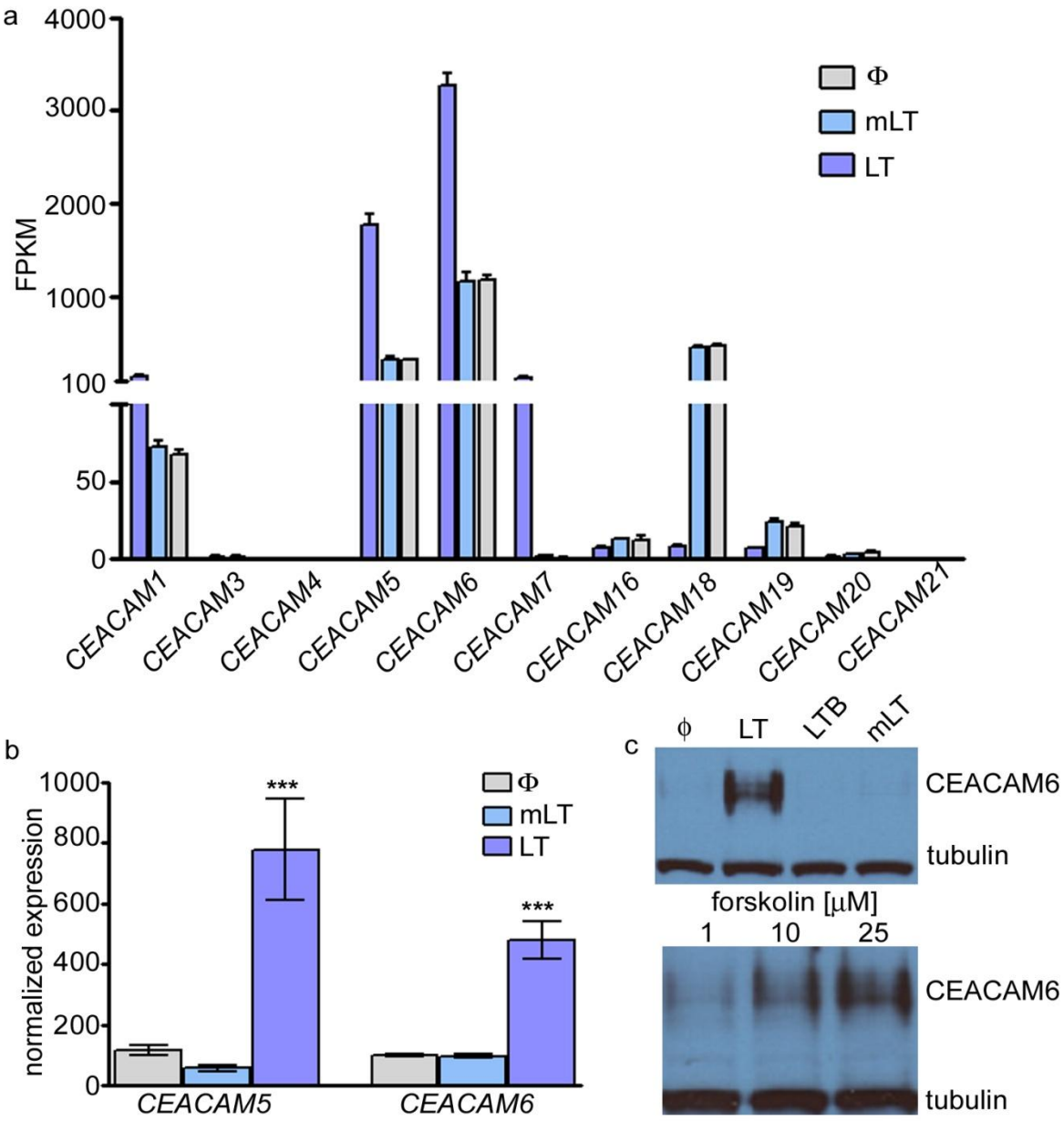


Figure 3. LT enhance intestinal CEACAMs expression.

a) Transcriptional profile of *CEACAMs* of intestinal cells treated with holotoxin (LT), mutant toxin (mLT) or untreated control (Φ). Bar represent mean FPKM value generated from RNAseq analysis with standard deviation of duplicate samples. b) Gene expression analysis of CEACAM5 and CEACAM6 by RT-PCR. Data represents % gene

expression normalized to *GAPDH* (n=5). Bar represent mean±SD. P values were calculated by nonparametric Mann-Whitney test. ***p<0.0001. c) Images shown are CEACAM6 from cells following treatment with LT, toxin B subunit (LTB), mLT or untreated (Φ), detected on immunoblot using anti-CEACAM6 monoclonal antibodies (top). Bottom image showing CEACAM6 from cells treated with different amount of forskolin. Tubulin was used as loading control.

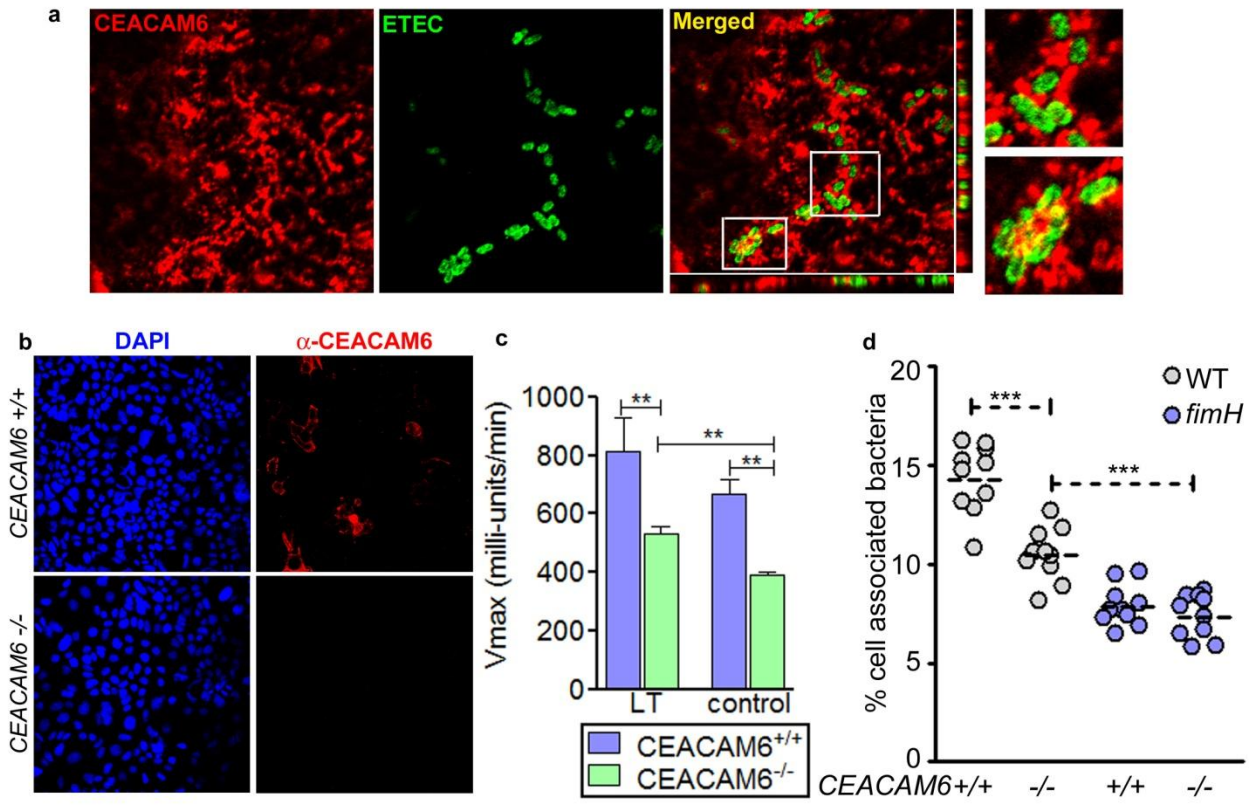


Figure 4. CEACAM6 expression enhance FimH mediated ETEC adhesion.

a) Confocal microscopic images of polarized culture of Caco-2 cells infected with WT ETEC, showing expression of CEACAM6 (anti-CEACAM6 monoclonal, red) and cell associated ETEC (anti-O78, green). Insets are magnified (6× digital) images showing induction of CEACAM6 expression around ETEC. b) Evaluation of *CEACAM6*$^{-/-}$ Caco-2 cells for the loss of CEACAM6 expression. Nuclei were stained with DAPI (blue) and α-CEACAM6 (red). c) GNA lectin biding to *CEACAM6*$^{-/-}$ cells following LT treatment. Bar represent mean±SD (n=5). P values were calculated by nonparametric Mann-Whitney test. **p<0.001. d) Adhesion of WT and *fimH mutant* to *CEACAM6*$^{-/-}$ cells. Horizontal

dashed line represent mean of duplicate experiments. P values were calculated by nonparametric Mann-Whitney test. ***p<0.0001.

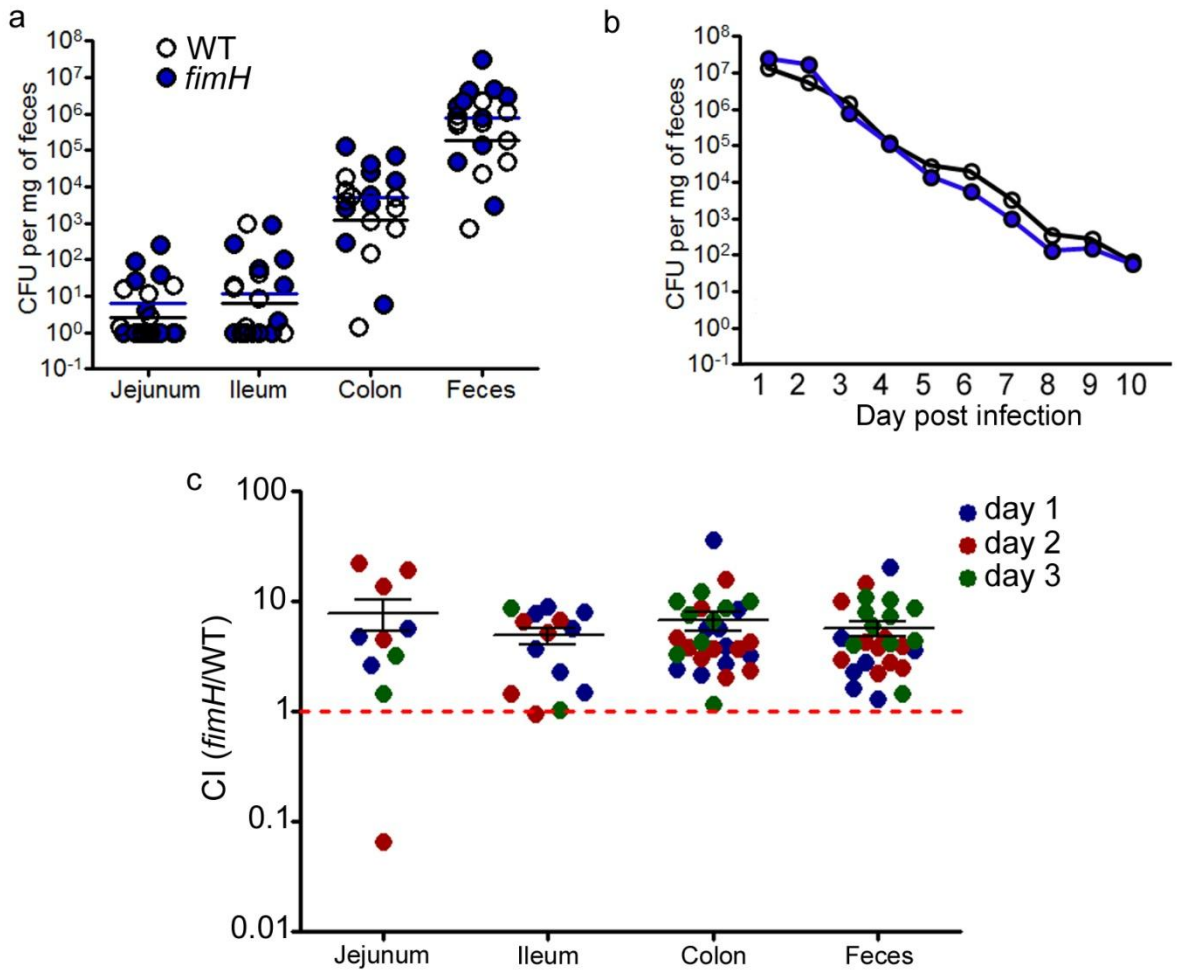


Figure 5. Dynamics of intestinal colonization of *fimH* mutants.

a) Comparison of mouse intestinal colonization of *fimH* mutants and WT ETEC at different niches 24 hrs post infection. Open circle represents WT and closed circle represents *fimH* mutant. Horizontal line represents geometric mean. b) Kinetics of

intestinal colonization evaluated by fecal shedding. Fecal specimens were collected every day for 10 days post infection for CFU count of *fimH* mutant and WT ETEC. Open circle represents WT and closed circle represents *fimH* mutant. c) Data represent competitive index (CI) of intestinal colonization of *fimH* mutant over WT. Mice co-infected with both strains were sacked at day 1 (n=10), day 2 (n=10) or day 3 (n=10) post infection for analysis of competitive colonization.

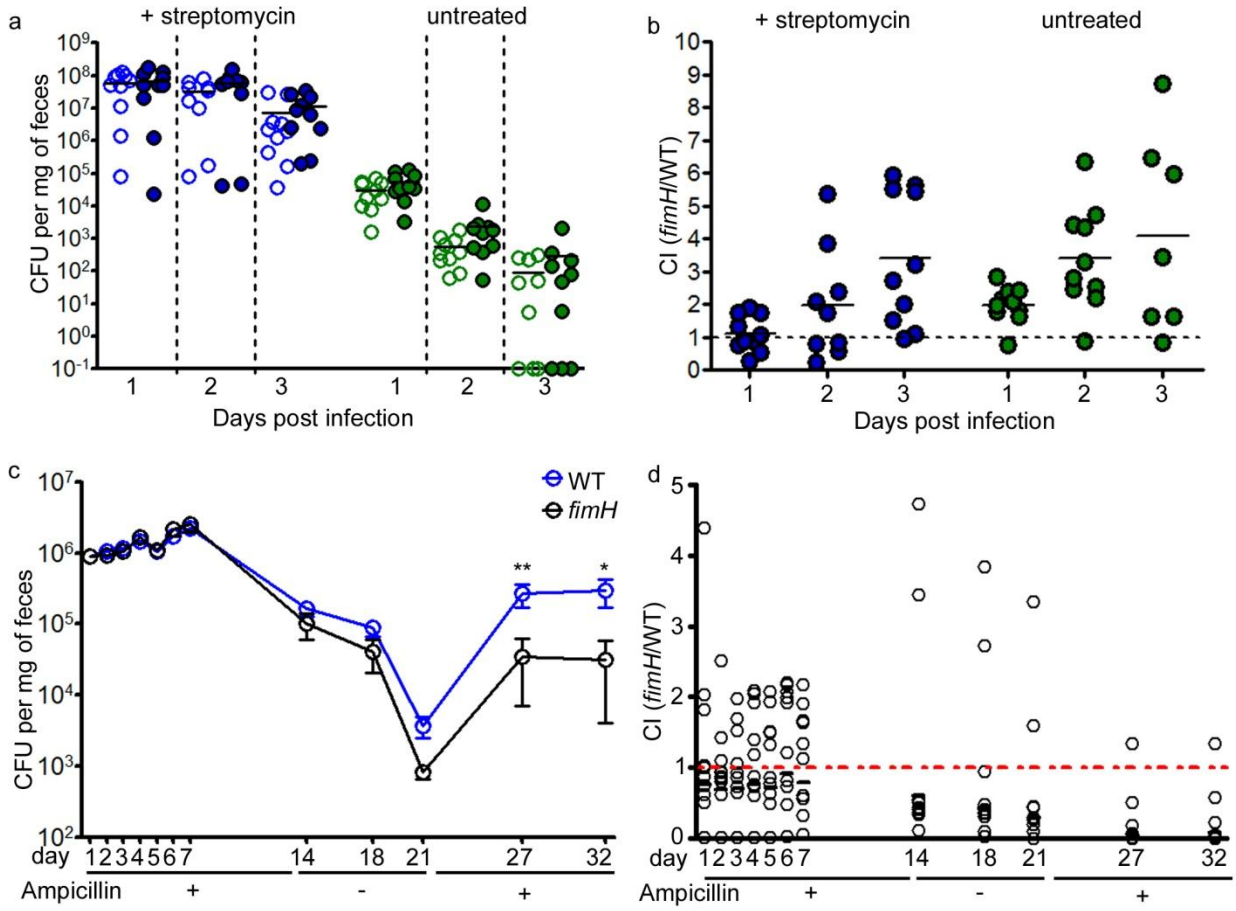


Figure 6. Antibiotic treatment affect FimH mediated colonization of ETEC in mice.

a) Graph showing fecal shedding of WT and *fimH* mutant from streptomycin treated mice (blue) and untreated mice (green). Data expressed as per mg of feces. Open circles represent data from WT and closed circles represent data from *fimH* mutant. Horizontal line represents geometric mean. b) Competitive index (CI) of intestinal colonization of *fimH* mutant over WT from mice treated with streptomycin (blue) and from untreated mice (green). c) Fecal shedding kinetics of WT and *fimH* mutant in the

presence or absence of ampicillin. Each circle represents mean±SEM of CFU per mg of feces from mice infected with WT (blue) or *fimH* mutants (black). P values were calculated by nonparametric Mann-Whitney test. *$p<0.05$ and **$p<0.001$. b) Competitive index (CI) of intestinal colonization of *fimH* mutant over WT from mice treated with streptomycin (blue) and from untreated mice (green). Dashed line represents CI of 1.

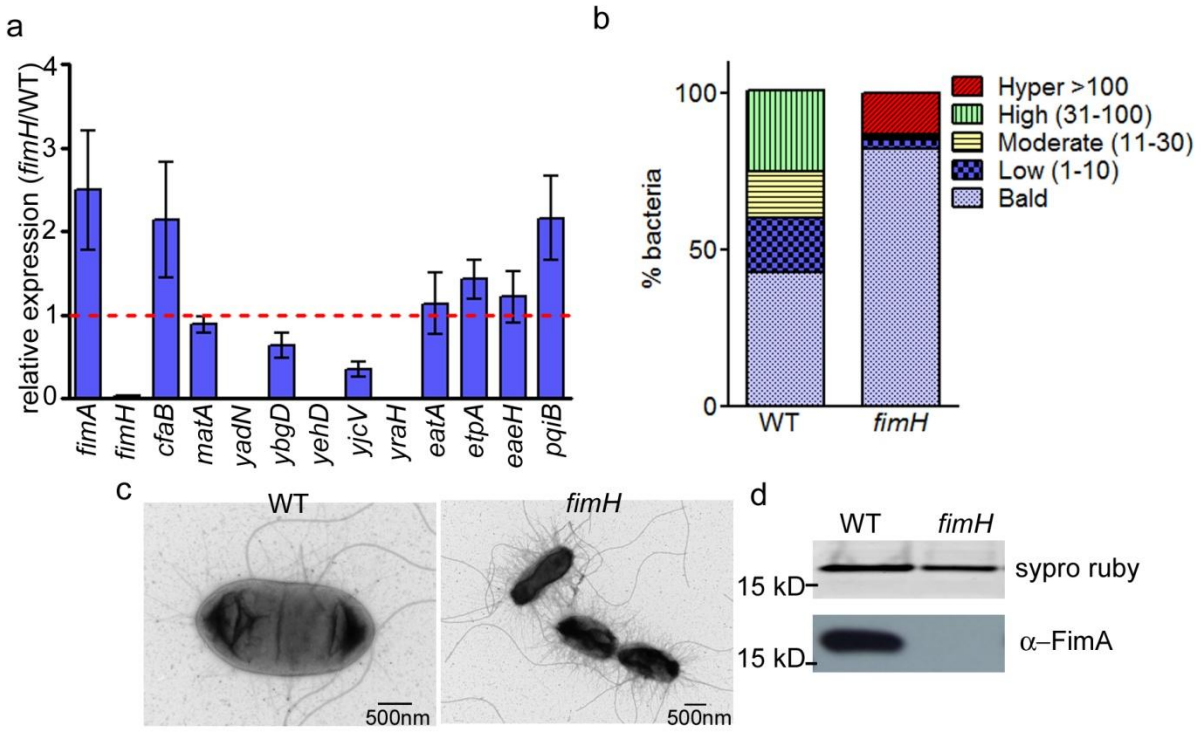


Figure 7. Altered expression of adhesion related genes in *fimH* mutants.

a) Transcription analysis of *fimH* mutants and WT ETEC collected from fecal samples. Each gene expression was normalized to housekeeping gene *arcA* and data presented as mean fold change. b) Evaluation of pili expression pattern of *fimH* mutants and WT ETEC counted under electron microscopy. At least 150 bacteria were evaluated per sample. c) Transmission electron microscopic images of WT ETEC and *fimH* mutants showing pili expression. d) Sypro ruby stained gel image of pili preparations from WT and *fimH* mutants ran on 4-15% gradient SDS-PAGE gel (top). Immunoblot of pili preparations from WT and *fimH* mutant showing anti-FimA reactivity to pili preparation from WT only (bottom).

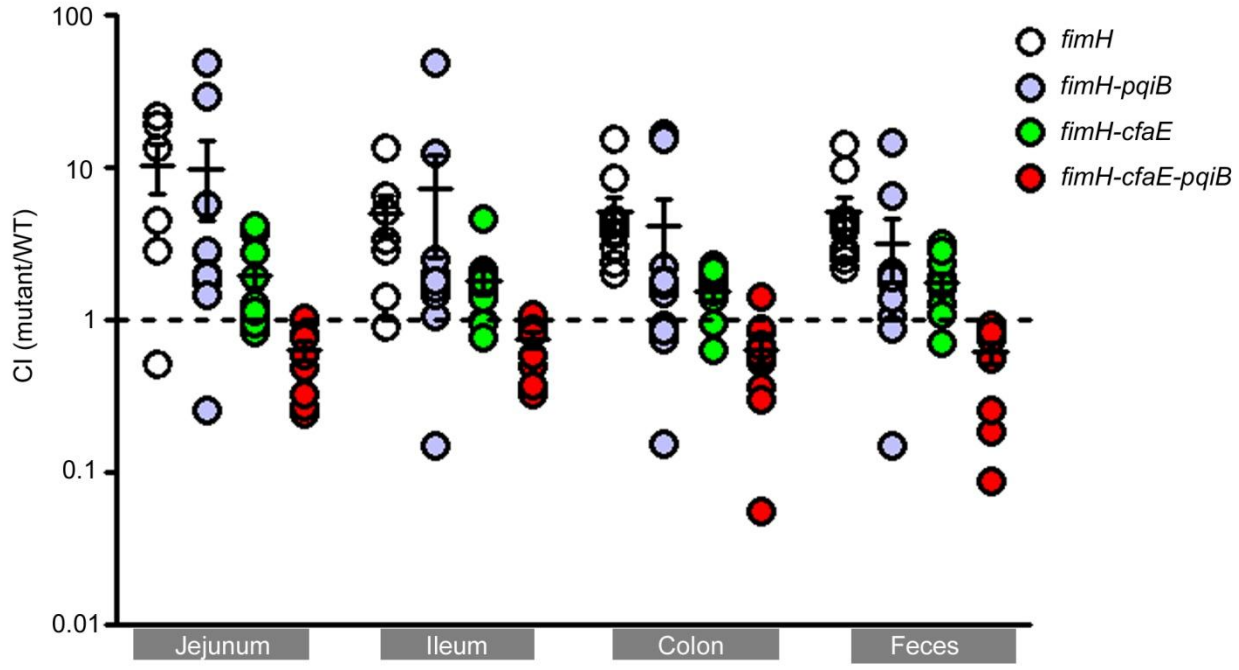


Figure 8. Mouse intestinal colonization of different mutants.

Competitive indices (CI) of *fimH* single mutant, *fimH-cfaE* and *fimH-pqiB* double mutant and *fimH-cfaE-pqiB* triple mutant vs WT ETEC. Each group of mice was co-infected with mutant and WT, and 24 hrs post infection mice were sacrificed for CFU count. Shown in plot is colonization to different intestinal niches, including fecal shedding. Each circle indicate datum from single mouse. Horizontal lines indicate mean CI (n=10 per group).

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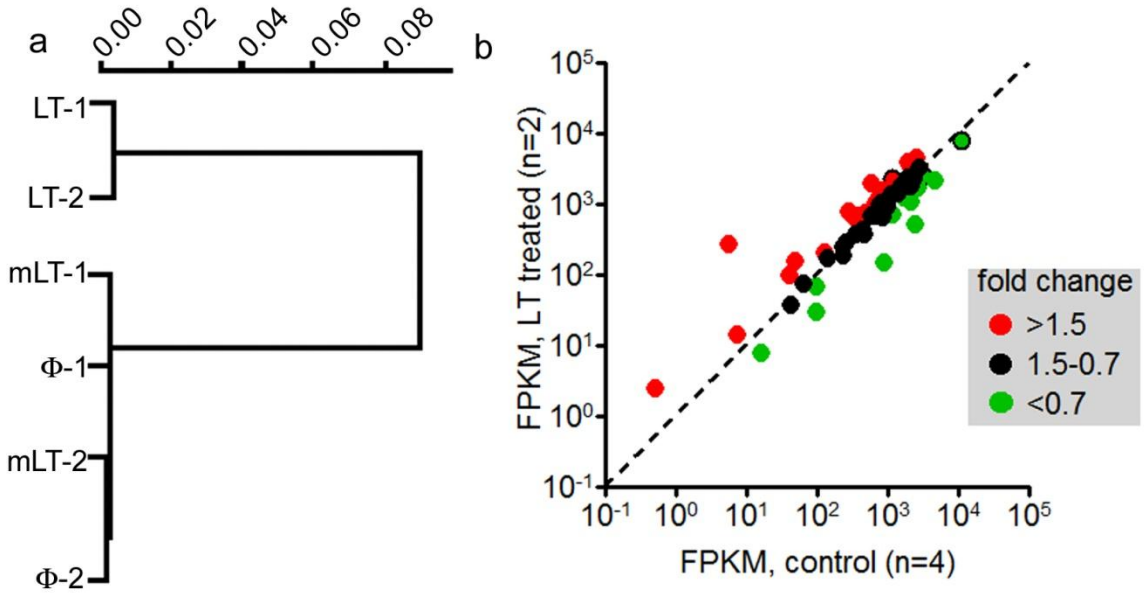
CHAPTER THREE: SUPPLEMENTARY DATA



S1 Figure. Differential expression of genes involved in glycosylation pathway.

a) Cluster analysis based on Pearson distance. Cells were treated with either holotoxin (LT) or mutant toxin (mLT) or untreated (Φ). Duplicate samples were run for each treatment. Cells treated with mLT and untreated cells were clustered together, therefore, used as control for differential expression analysis. b) Dot plot showing differential expression of genes involved in glycosylation process. Gene expression with more than 1.5 fold changes were shown in red, between 1.5-0.7 fold changes were shown in black and less than 0.7 fold changes were shown in green.

CHAPTER FOUR

Conclusion and Future Directions

4.1 Summary of the thesis

This thesis investigated the contribution of type 1 pili mediated pathogen-host interactions to ETEC pathogenesis. We found that FimH mediated interactions enhanced ETEC adhesion to intestinal cells and promoted efficient delivery of LT and ST enterotoxins (1). We also investigated host responses to LT in the context of pathogen-host interactions. In an ongoing collaborative effort, using RNAseq and mass spectrometric analyses, we showed that LT responses modified host glycosylation processes and enhanced expression of CEACAM6, a receptor of FimH. We also found that in response to deletion of *fimH* ETEC enhanced expression of other adhesion molecules, leading to increased intestinal colonization of mice.

These findings support a model in which FimH of type 1 pili interacts with mannosylated receptors of intestinal epithelium, promoting ETEC-host interactions and effective delivery of toxins that subsequently modulates host glycosylation processes and enhances CEACAM6 expression, changes which may further consolidate type 1 pili-mediated interactions with host (Fig 1). Overall, studies in this thesis corroborated the role of highly conserved type 1 pili of ETEC in pathogenesis and identified novel ETEC-host interactions.

4.2 Identification of an uncharacterized adhesion factor

Since *fimH* mutants were significantly more efficient in colonization of the murine intestine than WT ETEC, and the *pqiB* gene expression was elevated in the *fimH*

mutant strain, recovered from mouse feces, we speculated that increased expression of *pqiB* could contribute to colonization. To test this hypothesis, we examined both fecal shedding and mouse small intestinal colonization of an isogenic *pqiB* mutant (*pqiB*::CAT). Interestingly, in competition experiments, fecal shedding of the *pqiB* mutant was significantly diminished relative to WT ETEC (Fig 2a), a finding commensurate with impaired intestinal colonization of the intestine by the mutant (Fig 2b), suggesting that PqiB contributes to intestinal colonization by ETEC.

The *pqiB* gene was first found to be induced under oxidative stress, when Koh and Roe used a random promoter library screening technique to identify promoters induced with organic compound paraquat (2, 3). They identified a promoter upstream of an operon consisting of two genes named paraquat inducible A (*pqiA*) and B (*pqiB*) (2). They also showed that the promoter was under SoxRS regulon, as they detected decreased activity of the promoter in both *soxR* and *soxS* mutants (2). SoxRS may regulate expression of *pqiAB*, which encodes PqiA and PqiB, allowing *E. coli* to respond against oxidative stress they might encounter in the environment or inside the host (4-6). To investigate whether ETEC express PqiB, we first cloned the *pqiB* sequence of ETEC H10407 as in-frame with a *myc-his* tag sequence to construct p*pqiB-myc-his* expression plasmid, inserted in *E. coli* TOP10 for expression and purification of the protein. Recombinant PqiB-Myc-His protein expression was verified on anti-Myc immunoblotting (Fig 3a), and surface expression of Myc-tagged PqiB on TOP10 was detected by immunofluorescence microscopy (Fig 3b). Similarly, we detected PqiB expression in

ETEC H10407 grown in Luria broth (LB) (Fig 3c), however, H10407 growth in the presence of paraquat did not appear to enhance PqiB expression (Fig 3c). Deletion of *pqiB* did not have an appreciable effect on ETEC growth in LB (Fig 3d), and *pqiB* mutant and WT showed comparable growth in LB under different concentration of paraquat (Fig 3d). Despite increased expression of PqiB in *E. coli* K-12 following paraquat treatment (2), we were not able to replicate these responses in ETEC H10407, suggesting that *pqiB* of ETEC may respond to different signals.

Interestingly, we found that PqiB contains a mammalian cell entry (mce) domain first identified in *Mycobacterium tuberculosis* (7) where it facilitates pathogen host-interaction. Krachler *et al.* subsequently demonstrated that many Gram negative pathogens (8), including *Vibrio parahaemolyticus*, *Vibrio choleare* and enteropathogenic *E. coli* (EPEC), possess mce domain containing proteins (9), which they named multivalent adhesion molecules (MAM) (9, 10). They demonstrated that MAM7, which contain 7 mce domains, functions as an adhesin in *V. parahaemolyticus* as well as *V. cholerae* (9). EPEC also express MAM7 homologue of *Vibrio* encoded by *yebT*, and deletion of *yebT* decreased EPEC E2348/69 adhesion to host cells (9). Interestingly, we found that ETEC_1867 gene (*pqiB*) of ETEC H10407 encodes PqiB, which is 97% similar to *yebT*, and that the respective proteins are 99% similar (Supplementary figure 1). Therefore, we assumed that PqiB might also facilitate ETEC interaction with host, promoting adhesion.

Surprisingly, although episomal expression of *pqiB* of ETEC in *E. coli* TOP10 increased adhesion of TOP10 to Caco-2 human intestinal cells (Fig 4a), an isogenic *pqiB* ETEC mutant was significantly more adherent than WT ETEC to Caco-2 (Fig 4b), as well as to other intestinal cell lines (Fig 4c). The explanation for these seemingly contradictory adhesion phenotypes is not presently clear. Intriguingly, Nakayama and Zhang-Akiyama recently proposed that *pqiAB* operon encodes a transport pathway and contributes to membrane integrity (11), suggesting that it may serve other functions. Alternatively, the increased adhesion phenotype of the *pqiB* mutant could be the result of a compensatory increase in one or more other adhesin molecules. Further studies are needed to understand the contribution of PqiB, encoded on the core E. coli genome, to ETEC pathogenesis.

4.3 FimH variants

As the FimH adhesin of type 1 pili mediates interaction with host receptors via the lectin domain (12), minor changes to the FimH sequence could affect receptor-ligand interactions (13). For instance, variations in FimH sequence provide significant advantages to the bacteria during bladder (13, 14) and intestinal colonization (15). The mature FimH subunit is folded into two domains connected by 8 amino acid (aa) inter domain linker peptide: the N-terminal lectin domain (LD, 1-150 aa), containing the mannose binding pocket, and the C-terminal pilin domain (PD, 159-279 aa), interacting with the pilus rod to anchor the adhesin at the tip (12, 16-18). The mannose binding pocket is located at the top of the lectin domain, opposite the LD-PD interface (17), and

is accessible to the terminally exposed mannose sugars of the receptors. The binding pocket is comprised of 8 aa residues, including ^{1}Phe, ^{46}Asn, ^{47}Asp, ^{54}Asp, ^{133}Gln, ^{135}Asn, ^{140}Asp, and ^{142}Phe (16, 17). Changes in any one of these residues may reduce or completely abolish mannose binding activity (17). Additionally, interactions between the LD and PD domains alter the affinity of the mannose-binding pocket via an allosteric mechanism (19, 20). Moreover, multiple studies have shown that interdomain interactions of FimH regulate the conformational states- between a high affinity elongated state and a low affinity compact state (20-22). Switching between the elongated and compact conformations is important for proper binding to the receptor at appropriate niches. For instance, in the urinary tract, UPEC occasionally experience high shear force which separates the domains, causing the lectin domain to switch from a low affinity to a high affinity conformation (19). These high affinity interactions may prevent UPEC elimination from its niche. Consequently, changes in either of the domains or the linker could play an indirect role in modulating the mannose binding activity (23). In fact, mannose binding affinity is greatly enhanced in FimH variants with mutations in either the PD or LD which disrupt the interdomain interactions (19, 23, 24). Additionally, minor changes in FimH alter structural flexibility, allowing functional heterogeneity (25, 26).

Recently, we have sequenced 176 clinical isolates of ETEC recovered from symptomatic or asymptomatic fecal specimens (27). To examine the variation in FimH adhesins of ETEC, we performed sequence alignment analysis (CLUSTAL Omega,

version 1.2.4) of these isolates (table 1) and identified multiple variants of FimH (supplementary figure 2) that might impact type 1 pili mediated ETEC interaction with the receptor(s). Further studies will be necessary to evaluate the significance of these variants in ETEC infection.

4.4 Concluding remarks

This thesis work set out to better understand the contribution of highly conserved type 1 pili of ETEC, a genotypically and phenotypically diverse *E. coli* pathovar, to its interactions with intestinal epithelium. While some early studies predicted that type 1 pili mediate ETEC adhesion to intestinal epithelia (28, 29), others argued against it (30, 31). The findings described in the thesis convincingly demonstrate that type 1 pili of ETEC play an essential role in pathogenesis of these diverse bacteria. Additional findings, including detection of a novel FimH-CECAM6 mediated ETEC-host interaction, identification of the role of conserved PqiB in ETEC for intestinal colonization of mice, and detection of changes in glycosylation processes following LT treatment, suggest multitude of host responses to ETEC infection. In summary, documentation of role of type 1 pili and identification of at least two conserved factors that contribute to ETEC pathogenesis expand our understanding of the complex ETEC-host interactions. Comprehensive investigations of these emerging interactions could ultimately lead to broadly protective vaccine against highly diverse ETEC pathogens.

CHAPTER FOUR: REFERENCES

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Table 1: FimH variants of ETEC

Variants	Number of isolates (%)	Mutations at amino acid residues†																
		Signal peptide	Lectin domain‡									Pilin domain‡						
		6	5	9	23	27	66	67	69	118	119	165	195	202	210	243	248	269
	Common	T	T	T	N	A	G	V	S	V	A	A	Y	A	T	V	V	Q
Common	89 (50.57)																	
Variant #1	11 (6.25)	N																
Variant #2	1 (0.57)	N			T													
Variant #3	3 (1.70)	N									V							
Variant #4	1 (0.57)	N												V				
Variant #5	1 (0.57)	S																
Variant #6	1 (0.57)	P																
Variant #7	1 (0.57)		P															K
Variant #8	1 (0.57)			A									F					
Variant #9	2 (1.14)					V												
Variant #10	25 (14.20)						S							V				
Variant #11	6 (3.41)							G										
Variant #12	1 (0.57)							G										K
Variant #13	1 (0.57)								F									
Variant #14	1 (0.57)									G								
Variant #15	1 (0.57)											S						
Variant #16	1 (0.57)												D					
Variant #17	3 (1.70)													V				
Variant #18	11 (6.25)														I			
Variant #19	1 (0.57)															G		
Variant #20	1 (0.57)																G	
Variant #21	13 (7.39)																	K
Total	176																	

†Blank entries indicate identity with common variant
‡Amino acid number based on mature peptide

CHAPTER FOUR: FIGURES

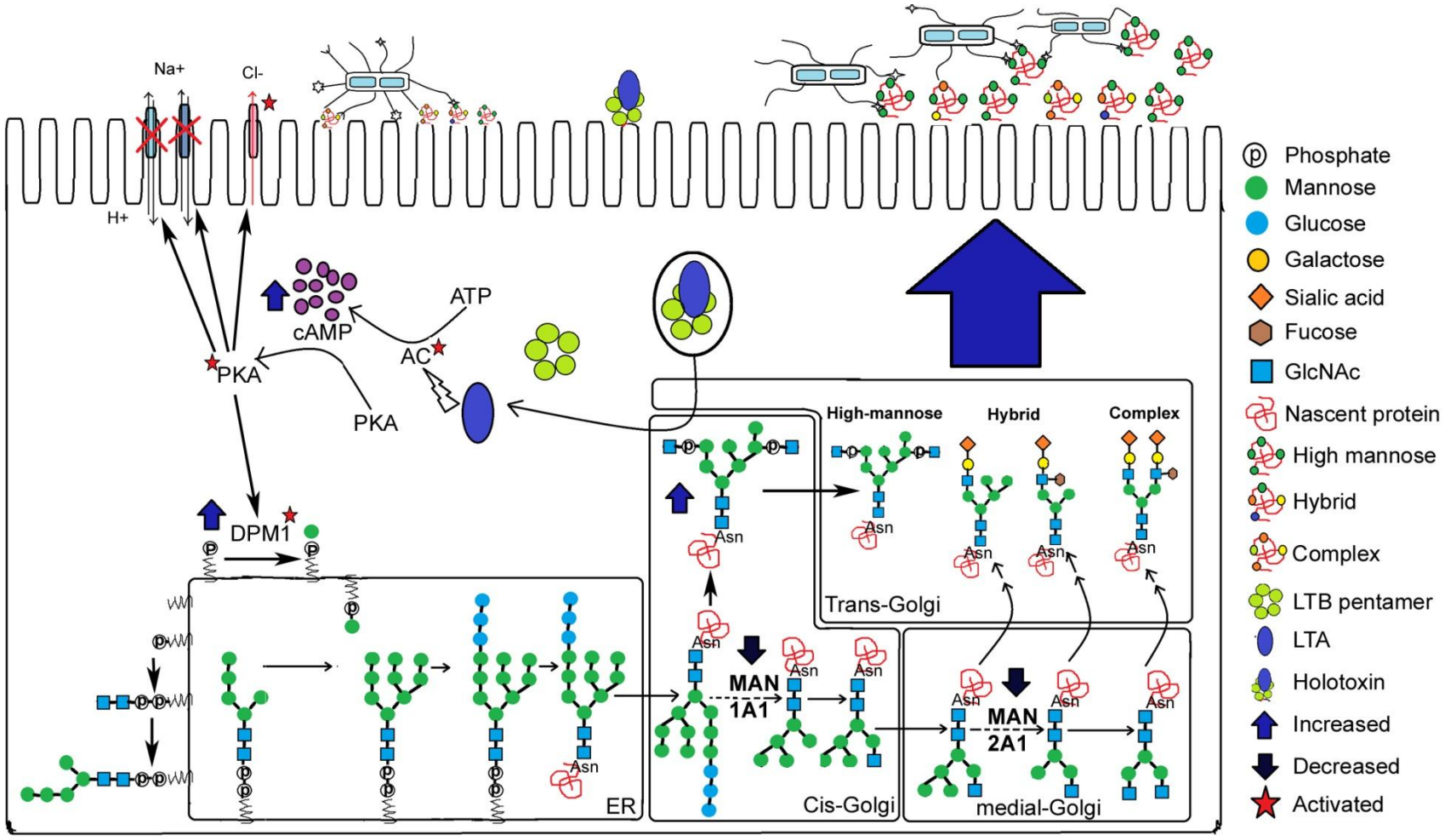


Figure 1: ETEC pathogenesis model.

Cartoon depicts emerging complexity of pathogen-host interactions following ETEC infection. ETEC interact with intestinal epithelium using multiple adhesion factors, including type 1 pili, for delivery of toxins. Following internalization, LT enhances intracellular cAMP, leading to modification of different host pathways, including multiple steps of the glycosylation process, resulting in alteration of cell surface glycan landscape that impact ETEC-host interactions. Enzymes those are activated in response to LT are marked with asterisks (red). Increased expressions are shown with upward arrows (green) and decreased expressions are shown with downward arrows (red).

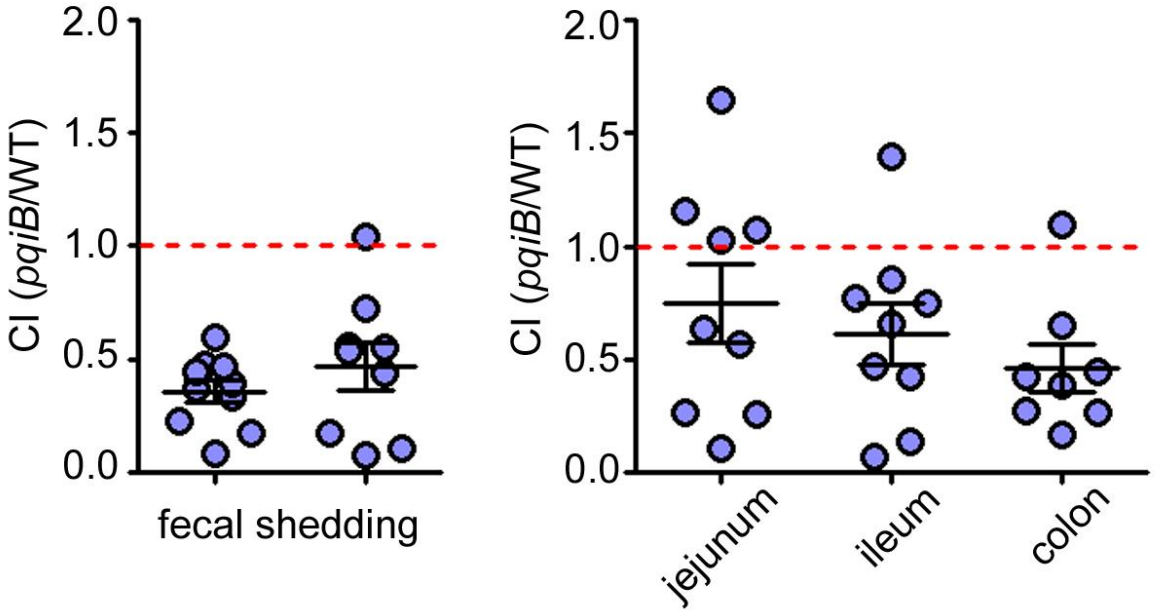


Figure 2: Mouse intestinal colonization by *pqiB* mutant.

Plot represents competitive index (CI) between *pqiB* mutant and WT in fecal shedding, acquired from duplicate experiments (left panel). Each dot represents datum from single mouse co-infected with *pqiB* mutant and WT ETEC. Competitive colonization index of *pqiB* mutant with WT in different intestinal niches, including jejunum, ileum and colon (right panel). Horizontal lines showing mean with SEM.

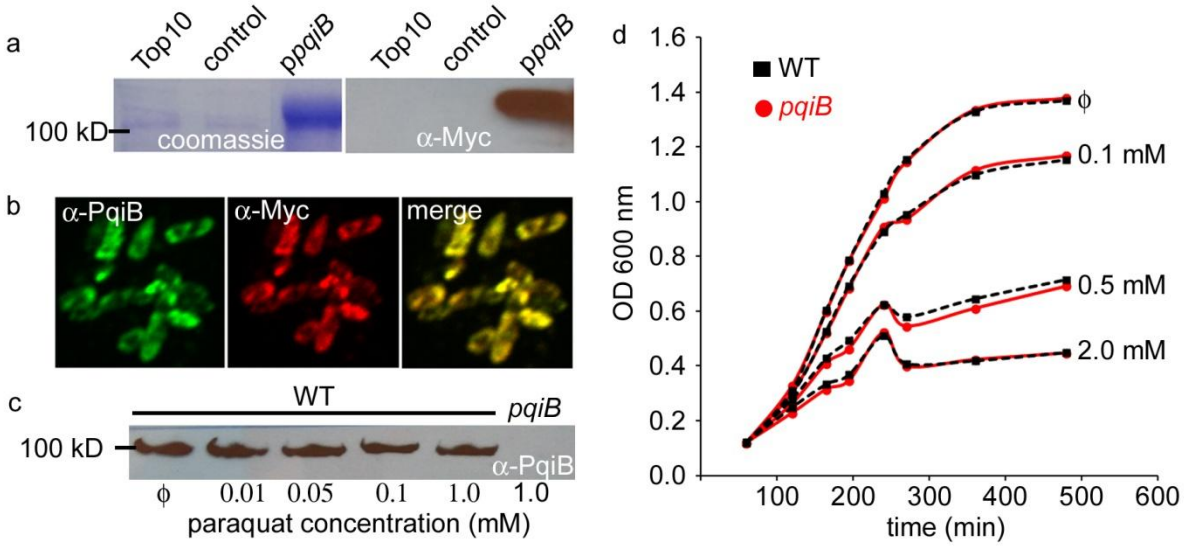


Figure 3: Detection of PqiB in ETEC and its contribution in growth.

a) Coomassie staining and anti-Myc immunoblot of PqiB of ETEC H10407 cloned into expression vector in fusion with Myc-Histidine tag and expressed in TOP10 *E. coli*. b) Confocal immunofluorescene microscopy images of TOP10 *E. coli* expressing Myc-His tagged PqiB. Signals were detected with affinity purified anti-PqiB antibodies (green) and anti-Myc monoclonal antibodies (red); merged image (right) shows co-localization of both antibodies. c) Anti-PqiB immunoblot of triton extracts of ETEC H10407 grown in different concentrations of paraquat. Triton extract of the *pqiB* mutant (right) is shown as a negative control. d) Growth curves of *pqiB* mutant and WT *E. coli* in different concentrations of paraquat.

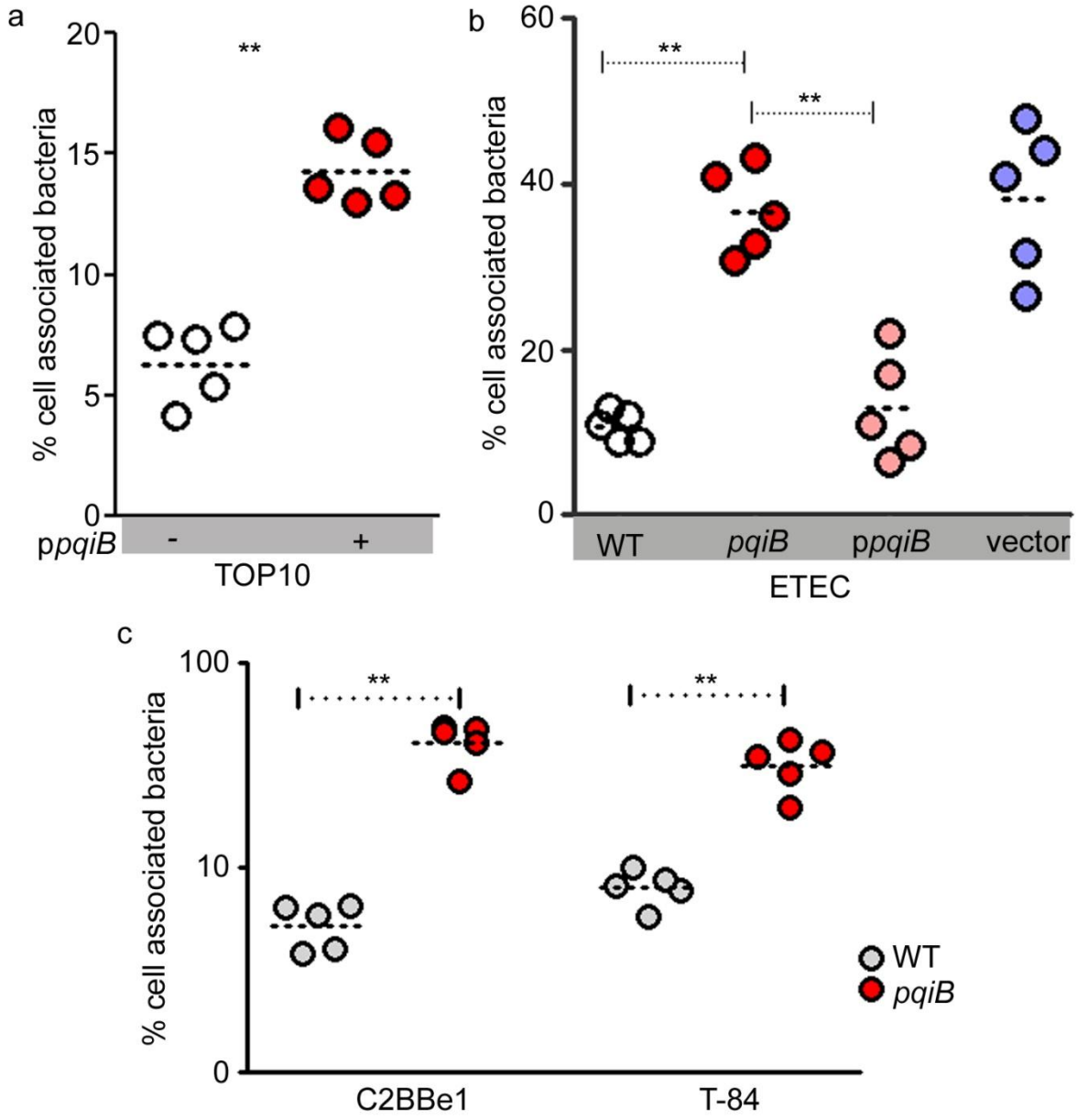


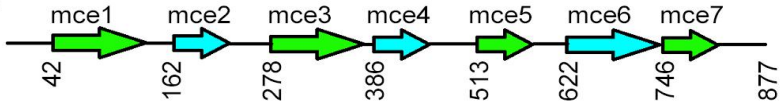
Figure 4: Contribution of PqiB in adhesion to intestinal cells.

a) *In vitro* adhesion assay of *E. coli* Top10 to Caco-2 intestinal cells in the absence or presence of PqiB of ETEC. Dashed lines represent mean adhesion. P value was

calculated by nonparametric Mann-Whitney test. **<0.001. b) Adhesion of isogenic H10407, *pqiB* mutant, complemented *pqiB* mutant (p*pqiB*) and vector control *pqiB* mutant ETEC to Caco-2 intestinal cells. Dashed lines represent mean adhesion. P values were calculated by nonparametric Mann-Whitney test. **<0.001. c) Adhesion of isogenic H10407 and *pqiB* mutant ETEC to other intestinal cell lines, including C2BBe1 and T-84 intestinal cells. Dashed lines represent mean adhesion. P values were calculated by nonparametric Mann-Whitney test. **<0.001.

CHAPTER FOUR: SUPPLEMENTARY DATA

a



b

```
ETEC   MSQETPASTTEAQIKNKRRISPFWLLPFIALMIAGWLIWDSYQDRGNTVTIDFMSADGIV 60
K-12   MSQETPASTTEAQIKNKRRISPFWLLPFIALMIASWLIWDSYQDRGNTVTIDFMSADGIV 60
EPEC   MSQETPASTTEAQIKNKRRISPFWLLPFIALMIAGWLIWDSYQDRGNTVTIDFMSADGIV 60
       ********************************.************************** 
ETEC   PGRTPVRYQGVEVGTVQDISLSDDLRKIEVKVSIKSDMKDALREETQFWLVTPKASLAGV 120
K-12   PGRTPVRYQGVEVGTVQDISLSDDLRKIEVKVSIKSDMKDALREETQFWLVTPKASLAGV 120
EPEC   PGRTPVRYQGVEVGTVQDISLSDDLRKIEVKVSIKSDMKDALREETQFWLVTPKASLAGV 120
       ************************************************************ 
ETEC   SGLDALVGGNYIGMMPGKGKEQDHFVALDTQPKYRLDNGDLMIHLQAPDLGSLNSGSLVY 180
K-12   SGLDALVGGNYIGMMPGKGKEQDHFVALDTQPKYRLDNGDLMIHLQAPDLGSLNSGSLVY 180
EPEC   SGLDALVGGNYIGMMPGKGKEQDHFVALDTQPKYRLDNGDLMIHLQAPDLGSLSSGSLVY 180
       ************************************************.*********** 
ETEC   FRKIPVGKVYDYAINPNKQGVVIDVLIERRFTDLVKKGSRFWNVSGVDANVSISGAKVKL 240
K-12   FRKIPVGKVYDYAINPNKQGVVIDVLIERRFTDLVKKGSRFWNVSGVDANVSISGAKVKL 240
EPEC   FRKIPVGKVYDYAINPNKQGVVIDVLIERRFTDLVKKGSRFWNVSGVDANVSISGAKVKL 240
       ************************************************************ 
ETEC   ESLAALVNGAIAFDSPEESKPAEAEDTFGLYEDLAHSQRGVIIKLELPSGAGLTADSTPL 300
K-12   ESLAALVNGAIAFDSPEESKPAEAEDTFGLYEDLAHSQRGVIIKLELPSGAGLTADSTPL 300
EPEC   ESLAALVNGAIAFDSPEESKPAEAEDTFGLYEDLAHSQRGVIIKLELPGGAGLTADSTPL 300
       ************************************************.*********** 
ETEC   MYQGLEVGQLTKLDLNPGGKVTGEMTVDPSVVTLLRENTRIELRNPKLSLSDANLSALLT 360
K-12   MYQGLEVGQLTKLDLNPGGKVTGEMTVDPSVVTLLRENTRIELRNPKLSLSDANLSALLT 360
EPEC   MYQGLEVGQLTKLDLNPGGNVTGEMTVDPSVVTLLRENTRIELRNPKLSLSDANLSALLT 360
       ******************:***************************************** 
ETEC   GKTFALVPGDGEPRKEFVVVPGEKALLHEPDVLTLTLTAPESYGIDAGQPLILHGVQVGQ 420
K-12   GKTFELVPGDGEPRKEFVVVPGEKALLHEPDVLTLTLTAPESYGIDAGQPLILHGVQVGQ 420
EPEC   GKTFELVPGDGEPRKEFVVVPGEKALLQEPDVLTLTLTAPESYGIDAGQPLILHGVQVGQ 420
       **** ********************:********************************** 
ETEC   VIDRKLTSKGVTFTVAIEPQHRELVKGDSKFVVNSRVDVKVGLDGVEFLGASASEWINGG 480
K-12   VIDRKLTSKGVTFTVAIEPQHRELVKGDSKFVVNSRVDVKVGLDGVEFLGASASEWINGG 480
EPEC   VIDRKLTSKGVTFTVAIEPQHRELVKGDSKFVVNSRVDVKVGLDGVEFLGASASEWINGG 480
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ETEC   IRILPGDKGEMKASYPLYANLEKALENSLSDLPTTTVSLSAETLPDVQAGSVVLYRKFEV 540
K-12   IRILPGDKGEMKASYPLYANLEKALENSLSDLPTTTVSLSAETLPDVQAGSVVLYRKFEV 540
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ETEC   GEVITVRPRANAFDIDLHIKPEYRNLLTSNSVFWAEGGAKVQLNGSGLTVQASPLSRALK 600
K-12   GEVITVRPRANAFDIDLHIKPEYRNLLTSNSVFWAEGGAKVQLNGSGLTVQASPLSRALK 600
EPEC   GEVITVRPRANAFDIDLHIKPEYRNLLTSNSVFWAEGGAKVQLNGSGLTVQASPLSRALK 600
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K-12   GAISFDNLSGASASQRKGDKRILYASETAARAVGGQITLHAFDAGKLAVGMPIRYLGIDI 660
EPEC   GAISFDNLSGASASQRKGDKRILYASETAARAVGGQITLHAFDAGKLAVGMPIRYLGIDI 660
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ETEC   GQIQTLDLITARNEVQAKAVLYPEYVQTFARGGTRFSVVTPQISAAGVEHLDTILQPYIN 720
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K-12   VEPGRGNPRRDFELQEATITDSRYLDGLSIIVEAPEAGSLGIGTPVLFRGLEVGTVTGMT 780
EPEC   VEPGRGNPRRDFELQEATITDSRYLDGLSIIVEAPEAGSLGIGTPVLFRGLEVGTVTGMT 780
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ETEC   LGTLSDRVMIAMRISKRYQHLVRNNSVFWLASGYSLDFGLTGGVVKTGTFNQFIRGGIAF 840
K-12   LGTLSDRVMIAMRISKRYQHLVRNNSVFWLASGYSLDFGLTGGVVKTGTFNQFIRGGIAF 840
EPEC   LGTLSDRVMIAMRISKRYQHLVRNNSVFWLASGYSLDFGLTGGVVKTGTFNQFIRGGIAF 840
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ETEC   ATPPGTPLAPKAQEGKHFLLQESEPKEWREWGTALPK 877
K-12   ATPPGTPLAPKAQEGKHFLLQESEPKEWREWGTALPK 877
EPEC   ATPPGTPLAPKAQEGKHFLLQESEPKEWREWGTALPK 877
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```
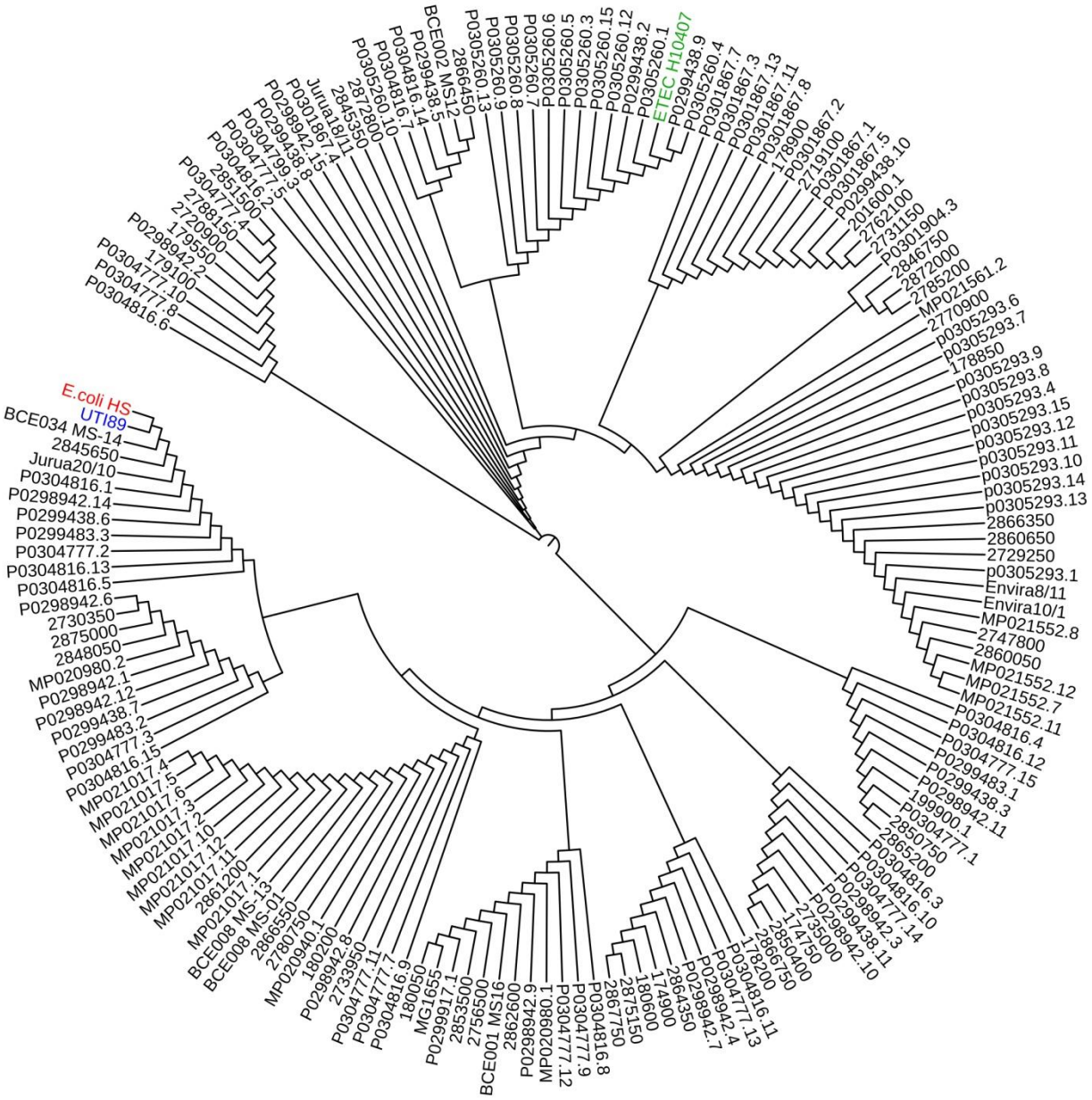

S1 Figure 1: Sequence alignment of mce domain containing proteins.

a) Schematic representation of mce domain containing protein (accession: CBJ01372) from H10407 (https://www.ncbi.nlm.nih.gov/protein/CBJ01372). mce domains (accession: 5UW8_A; https://www.ncbi.nlm.nih.gov/protein/5UW8_A) were identified using the domain-enhanced lookup time-accelerated BLAST (DELTA-BLAST). b) Data represent multiple sequence alignment of mce domain containing protein from ETEC strain H10407, commensal K-12 strain MG1655 (PqiB, NP_416348; https://www.ncbi.nlm.nih.gov/protein/NP_416348.2) and EPEC strain E2348/69 (YebT, WP_001347086; https://www.ncbi.nlm.nih.gov/protein/WP_001347086.1). Highlighted amino acid residues are sequences of multiple mce domains.



S2 Figure: Phylogenetic tree analysis of ETEC FimH adhesin.

FimH sequences were acquired from recently sequenced ETEC clinical isolates (27).

Phylogenetic tree was generated using iTOL software (http://itol.embl.de).

CURRICULUM VITAE

Alaullah Sheikh

EDUCATION:

08/2017	PhD in Molecular Microbiology and Microbial Pathogenesis, Division of Biology and Biomedical Science, Washington University in St. Louis
07**/**2001-07/2002 (exam. held in 2005)	M.S. in Biochemistry and Molecular Biology, University of Dhaka, Bangladesh
07/1997-07/2001 (exam. held in 2003)	B.S. in Biochemistry and Molecular Biology, University of Dhaka, Bangladesh

FELLOWSHIPS/TRAINING:

2011-2017	Graduate student, Molecular Microbiology and Microbial Pathogenesis Program, Division of Biology and Biomedical Sciences, Washington University in St. Louis, MO, USA
2005-2011	Research Investigator, Immunology and Centre for Vaccine Science, International Center for Diarrheal Disease Research, Bangladesh (icddrb), Dhaka, Bangladesh
2006-2010	Research Fellow, Global Infectious Disease Research Training Program in Vaccine Development, Fogarty International Center, U.S. National Institutes of Health
2006-2007	Research Scholar, Massachusetts General Hospital, Harvard Medical School, Boston, MA, U.S.A.

RESEARCH EXPERIENCE:

09/2011-	Washington University in St. Louis Advisor: James M Fleckenstein Investigation of the contribution of highly conserved features, including EaeH and type 1 pili, of enterotoxigenic *E. coli* (ETEC) in pathogen-host interactions.
08/2008- 08/2011	International Center for Diarrhoeal Disease Research, Bangladesh (icddrb) Advisor: Firdausi Qadri, PhD

1. Dissection of the host responses to *Salmonella* Typhi by comparing both cellular and humoral immune responses in children, young adult and adult patients.
2. Evaluation of the use of 'antigen specific antibodies in lymphocyte supernatant (ALS)' induced by infection with *Salmonella* Typhi as an immunological marker for detection of typhoid fever
3. Investigation of the cellular immune responses to different *Salmonella* Typhi proteins involved in pathogenesis.

09/2006-08/2008 Infectious Diseases, Massachusetts General Hospital
Advisor: Edward T Ryan

1. Expression profiling of in vivo induced *Salmonella* Typhi/Paratyphi-specific transcripts directly from the blood of infected patients.
2. Investigating the genomics of *Salmonella* Typhi and *Salmonella* Typhimurium *in vitro* using high throughput DNA microarray to screen candidate genes involved in pathogenesis.

01/2004-08/2006 International Center for Diarrhoeal Disease Research, Bangladesh (icddrb)
Advisor: Firdausi Qadri, PhD

Characterization of the innate and adaptive cellular immune responses to zinc supplementation in children infected with enterotoxigenic *Escherichia coli* (ETEC).

AWARDS/DISTINCTIONS:

1. Merit Scholarship 2002 for the academic excellence in MS, University of Dhaka.
2. Merit Scholarship 2001 for the academic excellence in BS Honors, University of Dhaka.
3. Merit Scholarship for academic excellence in Secondary and Higher Secondary Education, Board of Education, Jessore, Bangladesh
4. Travel Awards, U.S.-Japan Cooperative Medical Science Program (CMSP): 13th International Conference on Emerging Infectious Diseases in the Pacific Rim – Focused on Enteric Diseases, April 6 - 9, 2009, India.

5. Travel Awards, ASM conference on Salmonella: Biology, Pathogenesis and Prevention, October 5-9, 2009, Aix-en-Provence, France

PATENTS:

Ryan ET, Charles RC, **Sheikh A**, Qadri F. *Kits and assays for amplification of expressed salmonella genes from blood.* Pub. No.: WO/2012/071405 Publication Date: 31.05.2012; http://patentscope.wipo.int

TEACHING, SUPERVISION, MENTORSHIP:

Spring 2013	Washington University in St. Louis Teaching assistant Biology: Laboratory experiments with eukaryotic microbes
2008 to 2011	International Center for Diarrhoeal Disease Research, Bangladesh (icddrb) Project supervision: M. Sc. and M. Phil. students from the department of Biochemistry and Molecular Biology, University of Dhaka and BRAC University, Bangladesh; and Fellow from Harvard HIGH iSURF.

PUBLICATIONS:

1. **Sheikh A**, Rashu R, Begum YA, Kuhlman FM, Ciorba MA, Hultgren SJ, Qadri F, Fleckenstein JM. Highly conserved type 1 pili promote enterotoxigenic E. coli pathogen-host interactions. PLoS Negl Trop Dis. 2017;11(5): e0005586. PMID: 28531220
2. Jason W. Sahl, Jeticia R. Sistrunk, Nabilah Baby, Yasmin Begum, Qingwei Luo, **Alaullah Sheikh**, Firdausi Qadri, James M. Fleckenstein, David A. Rask. Insights into enterotoxigenic Escherichia coli diversity in Bangladesh utilizing genomic epidemiology. Scientific Reports 7, 2017; Article number: 3402, doi:10.1038/s41598-017-03631-x. PMID: 28611468
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4. Examination of the Enterotoxigenic Escherichia coli Population Structure during Human Infection.Sahl JW, Sistrunk JR, Fraser CM, Hine E, Baby N, Begum Y, Luo Q, **Sheikh A**, Qadri F, Fleckenstein JM, Rasko DA. MBio. 2015 Jun 9;6(3):e00501. PMID: 26060273

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8. Fleckenstein JM, **Sheikh A**. Designing vaccines to neutralize effective toxin delivery by enterotoxigenic Escherichia coli. Toxins (Basel). 2014 Jun. PMID:24918359
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11. Luo Q, Kumar P, Vickers T, **Sheikh A**, Lewis WG, Rasko DA, Sistrunk J, Fleckenstein JM.Enterotoxigenic Escherichia coli secrete a highly conserved mucin-degrading metalloprotease to effectively engage intestinal epithelial cells. Infect Immun. 2013 Nov 18. PMID: 24246556
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Larocque RC. Natural selection in a bangladeshi population from the cholera-endemic ganges river delta. Sci Transl Med. 2013 Jul 3;5(192):192ra86. doi: 10.1126/scitranslmed.3006338. PMID:23825302

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MEETING / CONFERENCE:

1. **Alaullah Sheikh,** Rita Kansal, Rasheduzzaman, Yasmin Ara Begum, Scott J Hultgren, Firdausi Qadri, and James M Fleckenstein. *Type 1 fimbriae promote enterotoxigenic E. coli adhesion to intestinal epithelial cells and are required for optimum virulence.* Meeting on Microbial Pathogenesis and host response, September 8-12, 2015, Cold Spring Harbor Laboratory
2. **Alaullah Sheikh**, Rita Kansal, Nabilah Ibnat, Yasmin Ara Begum, Jerome S Pinkner, Scott J Hultgren, Firdausi Qadri, and James M Fleckenstein. *Highly conserved type 1 fimbriae promote enterotoxigenic Escherichia coli (ETEC) adhesion and enterotoxin delivery.* Global Health and Infectious Disease conference-2014, Washington University in St. Louis
3. **Alaullah Sheikh**, Md. Saruar Bhuiyan, Tania Sultana, Lillian Tsai, Farhana Khanam, Md. Abu Sayeed, Fahima Chowdhury, Amit Saha, Jason B Harris, W. Abdullah Brooks, Stephen B. Calderwood, Alejandro Cravioto, Edward T. Ryan, and Firdausi Qadri. *Rapid diagnosis of typhoid fever in patients using a simplified "antibody in lymphocyte supernatant (ALS)" technique and its application in field settings*. Presented in the 14th U.S.-Japan Cooperative Medical Sciences Program (CMSP) Regional Conference on "Emerging Infectious Diseases in the Pacific Rim (EID): Diagnostics" in Penang, Malaysia from October 4–6, 2010.
4. Richelle C. Charles, **Alaullah Sheikh**, Bryan Krastins, Jason B. Harris, Md. Saruar Bhuiyan, Regina C. LaRocque, Tanya Logvinenko, David A. Sarracino, Indira Kudva, Jana Eisenstein, Michael Podolsky, Anuj Kalsy, Albrecht Ludwig,

Manohar John, Stephen B. Calderwood, Firdausi Qadri, Edward T Ryan; *Immunoaffinity proteomic-based screening of antibody responses during wild type S. Typhi infection in humans* at DMID International Research in Infectious Diseases Meeting, May 18-20, 2010.

5. **Alaullah Sheikh**, Richelle C. Charles, Sean Rollins, Jason B. Harris, Md. Saruar Bhuiyan, Farhana Khanam, Archana Bukka, Anuj Kalsy, Steffen Porwollik, W. Abdullah Brooks, Regina LaRocque, Michael McClelland, Tanya Logvinenko, Alejandro Cravioto, Stephen B. Calderwood, James E. Graham, Firdausi Qadri, Edward T. Ryan. *High throughput gene expression profiling of Salmonella enterica serovar Paratyphi A in the blood of bacteremic patients in Bangladesh*. Presented in 3rd ASM conference on Salmonella: biology, pathogenesis and prevention-2009, France; Page-16; ISBN: 978-1-55581-544-8.
6. **Alaullah Sheikh**, Farhana Khanam, Taibur Rahman, Martin Pacek, Yanhui Hu, Andrea Baresch, Md. Saruar Bhuiyan, Sean Rollins, Robert Citorik, Anuj Kalsy, Richelle Charles, Regina C. LaRocque, Joshua LaBaer, Stephen B. Calderwood, Jason B. Harris, Firdausi Qadri, Edward T. Ryan; "*Evaluation of interferon-γ responses in patients with Salmonella enterica serovar Typhi bacteremia in Dhaka, Bangladesh*" presented at ASTMH meeting-2009; 300 www.astmh.org
7. Jason B. Harris, Richelle C. Charles, Lauren M. Lebrun, Michael Chase, **Alaullah Sheikh**, Regina C. Larocque, Brian Krastins, David A. Sarracino, Abdullah Tarique, Stephen B. Calderwood, Elizabeth L. Hohmann, Firdausi Qadri, Kenneth Parker, Edward T. Ryan; "*Proteomic analysis of the PhoP regulon in Salmonella enterica serovars Typhi and Typhimurium*" at DMID International Research in Infectious Diseases Meeting, 2008.

INVITED TALK:

1. *Type 1 fimbriae promote ETEC adhesion and are required for optimal virulence.* Presented in the 18th International Conference on "Emerging Infectious Diseases (EID) in the Pacific Rim". This conference will be held on January 11-12, 2016 at the Bethesda North Marriott Hotel and Conference Center in Bethesda, Maryland.
2. *Highly conserved type 1 fimbriae promote enterotoxigenic Escherichia coli (ETEC) adhesion and enterotoxin delivery.* Presented in the United States-Japan Cooperative Medical Science Program (CMSP) *16th* Regional Conference on February 9-15, 2014 at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B).
3. *High throughput gene expression profiling of Salmonella enterica serovar Paratyphi A in the blood of bacteremic patients in Bangladesh.* Presented in 3rd

ASM conference on Salmonella: biology, pathogenesis and prevention-2009, France; Page-16; ISBN: 978-1-55581-544-8.